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| (54) Title: TETRACYCLINE REPRESSOR-MEDIATED BINARY REGULATION SYSTEM FOR CONTROL OF GENE EXPRESSION IN TRANSGENIC ANIMALS (57) Abstract The present invention relates to a tetracycline repressor-mediated binary regulation system for the control of gene expres- sion in transgenic animals. It is based, at least in part, on the discovery that, in a non-human transgenic animal that carries a first transgene under the control of a modified promoter comprising a tetR operator sequence and a second transgene encoding the tetR repressor protein, expression of the first transgene may be efficiently induced by administering tetracycline to the animal. | | |

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**TETRACYCLINE REPRESSOR-MEDIATED BINARY
REGULATION SYSTEM FOR CONTROL OF
GENE EXPRESSION IN TRANSGENIC ANIMALS**

5

1. INTRODUCTION

The present invention relates to a tetracycline repressor-mediated binary regulation system for the control of gene expression in transgenic animals. It is based, at least in part, on the discovery that, in
10 a non-human transgenic animal that carries a first transgene under the control of a modified promoter comprising a tetR operator sequence and a second transgene encoding the tetR repressor protein, expression of the first transgene may be efficiently
15 induced by administering tetracycline to the animal.

2. BACKGROUND OF THE INVENTION

**2.1. CONTROL OF GENE EXPRESSION
IN TRANSGENIC ANIMALS**

20 The production of transgenic animals for both experiment and agricultural purposes is now well known (Wilmut et al., 7 July 1988, New Scientist pp. 56-59). In research, transgenic animals are a powerful tool that have made significant contributions to our
25 understanding of many aspects of biology and have contributed to the development of animal models for human diseases (Jaenisch, 1988, Science 240:1468-1474). It is also clear that several livestock species can be made transgenic and these species
30 promise to expand and revolutionize the method of production and diversity of pharmaceutical products available in the future, in addition to improving the agricultural qualities of the livestock species (Wilmut et al., supra).

35 A critical, often neglected, aspect of developing transgenic animals is the process whereby expression

of the newly introduced gene, referred to as the transgene, is controlled. This is an important process since stringent regulation of transgene
5 expression is often important both for practical, regulatory and safety reasons and to maintain the health of the transgenic animal. In the past either "inducible" or "tissue specific" regulatory mechanisms have been used. Inducible regulation is defined
10 herein as a method of gene regulation which allows for some form of outside manipulation of the onset and/or level of transgene expression. Tissue specific regulation is defined herein as a method for targeting transgene expression to particular tissues or organs.

15 Inducible gene regulation may be achieved using relatively simple promoter systems such as the metallothionein heat shock promoters, or by using promoters which are responsive to specific compounds such as the Mouse mammary tumor virus LTR which is
20 responsive to glucocorticoid stimulation. More flexible, though more complex inducible regulation systems can be achieved through a "binary" gene approach which utilizes a transactivator gene product to control expression of a second gene of interest.
25 Tissue specific gene regulation usually consists of simple single gene methods (Byrne et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5473-5477; Ornitz et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:698-702), although binary transactivator systems can also
30 provide a high degree of tissue specificity.

These current systems provide only a limited ability to control the time of transgene expression within individual animals. In this respect tissue specific promoter elements provide no method to
35 control the onset of transgene activity, but function merely to target gene expression to defined sites.

Simple inducible promoters such as metallothionein generally lack tissue specificity and usually have some aspect of endogenous basal expression which cannot be controlled. Thus even for the extensively used inducible metallothionein promoter this approach at best only permits selection of the time at which a relative increase in transgene expression can be induced.

Binary transactivation systems typically consist of two transgenic animals. One animal contains the gene of interest controlled by a promoter element that requires a specific transactivator gene product for expression. Thus, the gene of interest is not expressed in the absence of the transactivator. A second transgenic animal is then made which expresses the required transactivator in the desired tissue. By mating these two transgenic animals, offspring containing both the gene of interest and the transactivator transgene can be produced. Only in these doubly transgenic animals is the gene of interest expressed. Since expression of the gene of interest requires the transactivator, this binary approach dramatically reduces or eliminates any undesirable basal expression inherent in simple inducible systems. Additionally, if expression of the transactivator is targeted using a tissue specific promoter, then in the double transgenics, expression of the gene of interest is in effect targeted to the same specific tissue. Binary systems provide therefore a low resolution method of temporal regulation in as much as they allow the determination of which generation of animals will express the gene of interest. These systems provide little ability, however, to control the time and level of gene expression within an individual transgenic animal.

For many applications it is necessary to accurately control the time and pattern of transgene expression within an individual transgenic animal.

5 For example, many attempts have been made to produce transgenic pigs which express increased levels of growth hormone (Vize et al., 1988, J. Cell Sci. 90:295-300;; Pinkert et al., 1990, Dom. Animal Endocrinol. 7:1-18). Elevated growth hormone levels

10 dramatically decrease the amount of body fat in pigs, and increase the animals overall feed efficiency. These effects would be beneficial, both to the consumer who could purchase a leaner, healthier product, and to the producer who can profit from

15 having a more efficient animal. To date however, all attempts to increase the level of growth hormone through production of transgenic pigs have also produced serious pathological conditions which greatly reduce the health of the animals. These pathologies

20 are the direct result of uncontrolled, constitutive expression of growth hormone, since many studies using exogenous hormone administration for short periods of time have not produced pathologies, while still benefiting feed efficiency and fat content. In this

25 situation, a regulatory method to control onset and level of expression from a growth hormone transgene would be extremely useful.

2.2. REPRESSOR-MEDIATED GENE CONTROL

30 Transcriptional repressors are usually allosteric DNA binding proteins with at least two functional sites. One site on the protein is used to bind DNA. The DNA binding site binds to a defined DNA sequence which is known as the operator site. Operator sites

35 usually consist of palindromic sequences of 12 or more base pairs. A gene which is regulated by a repressor

of the newly introduced gene, referred to as the transgene, is controlled. This is an important process since stringent regulation of transgene
5 expression is often important both for practical, regulatory and safety reasons and to maintain the health of the transgenic animal. In the past either "inducible" or "tissue specific" regulatory mechanisms have been used. Inducible regulation is defined
10 herein as a method of gene regulation which allows for some form of outside manipulation of the onset and/or level of transgene expression. Tissue specific regulation is defined herein as a method for targeting transgene expression to particular tissues or organs.

15 Inducible gene regulation may be achieved using relatively simple promoter systems such as the metallothionein heat shock promoters, or by using promoters which are responsive to specific compounds such as the Mouse mammary tumor virus LTR which is
20 responsive to glucocorticoid stimulation. More flexible, though more complex inducible regulation systems can be achieved through a "binary" gene approach which utilizes a transactivator gene product to control expression of a second gene of interest.
25 Tissue specific gene regulation usually consists of simple single gene methods (Byrne et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5473-5477; Ornitz et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:698-702), although binary transactivator systems can also
30 provide a high degree of tissue specificity.

These current systems provide only a limited ability to control the time of transgene expression within individual animals. In this respect tissue specific promoter elements provide no method to
35 control the onset of transgene activity, but function merely to target gene expression to defined sites.

must have at least one operator site located within its promoter/regulatory region. A second site on the repressor protein binds a specific ligand, usually a small macromolecule such as an amino acid, sugar, or antibiotic. When the ligand is bound to the repressor, it causes a conformational shift such that the affinity of the repressor for the operator sequence is greatly reduced. For this reason, the ligand is frequently referred to as the "inducer", since it causes the repressor to disassociate from the operator, thereby eliminating the repressor's effect and allowing expression of the gene.

Only the bacterial repressors LacI, LexA and tetR have been shown to function in mammalian (LacI and LexA) or plant (tetR) tissue culture cells. The first report of utilizing bacterial repressors in eukaryotes was from Brent and Ptashne who showed that LexA could function in yeast (1984, Nature 312:612-615). Subsequently, both LexA and LacI have been shown to function in mammalian tissue culture systems (Smith et al., 1988, EMBO J. 7:3975-3981). Of these repressors LacI has been most extensively studied. For LacI repression, single or multiple operator sites have been positioned in three major locations: (i) between the transcription start site and the first codon of the mRNA; (ii) between the TATA-box sequence and the transcription start site; and (iii) between the TATA-box sequence and any more distal regulatory signal sequences. These studies reveal two predominant results. First, operators located in all three positions were effective in rendering the modified promoter subject to LacI repression. Second, the presence of multiple operator sequences allowed greater levels of repression than did single operator insertions. From these studies it appears the LacI

administering tetracycline to a non-human transgenic animal that carries a first transgene, which is the gene of interest under the control of a promoter
5 modified to comprise a tetR operator sequence and a second transgene encoding the tetR repressor protein.

The present invention offers the advantage that, in the absence of tetracycline, expression of the gene of interest occurs at only very low levels due to
10 efficient repression by tetR. In preferred, non-limiting embodiments of the invention, repression by tetR is further enhanced by utilizing a synthetic tetR gene which is devoid of splice signals and has optimized codon usage for mammalian cells.
15 Accordingly, the present invention allows tight control of gene expression in transgenic animals by withholding or administering tetracycline.

4. DESCRIPTION OF THE FIGURES

- 20 Figure 1. A. Nucleotide sequence of tetR operator as it occurs in Tn10, and in the oligonucleotides used to produce the modified PEPCK promoter elements. Bold face lettering represent the OP1 and OP2 tetR binding sites. The general purpose
25 oligonucleotide is the sequence from p $\delta\delta 7$. The flanking EcoRI and AccI restriction sites used to excise this operator sequence are indicated. Additional restriction sites present in the plasmid, but not indicated here, which can be
30 used to excise the operator include PstI, BamHI, SpeI, SbaI, NotI, EagI, SacII, BstXI, and SacI on the 5' side and XhoI, ApaI and KpnI on the 3' side. The sequence of the PEPCK-TATA box operator is also indicated (see methods).
35 Figure 1. B. Nucleotide sequence of the $\delta\delta 7$

original tetR responsive CAMV promoter, in which the operator sites flank the TATA-box into transgenic tobacco plants. Unexpectedly, this promoter, which exhibited very good regulation in tissue culture assays was not very effective in regulating gene expression in transgenic plants. Instead they found that effective repression and induction in transgenic plants occurred when the operator sites were positioned just downstream of the normal transcription start site.

3. SUMMARY OF THE INVENTION

The present invention relates to a tetracycline repressor-mediated binary regulation system for the control of gene expression in non-human transgenic animals. It is based, at least in part, on the discovery that in transgenic mice carrying two transgenes, the first encoding bovine growth hormone (bGH) under the control of a PEPCK promoter modified to comprise the tetR operator sequence at the NheI site, and the second encoding tetR repressor protein under the control of an unmodified PEPCK promoter, expression of bGH could be efficiently and selectively induced by administering tetracycline to the transgenic mice.

In particular embodiments, the present invention provides for (i) animal promoter elements modified to comprise a tetR operator sequence; (ii) nucleic acid molecules comprising a gene of interest under the control of such a modified promoter; (iii) non-human transgenic animals that carry a transgene under the control of said modified promoter and/or a transgene encoding the tetR repressor protein; and (iv) a method of selectively inducing the expression of a gene of interest in a non-human transgenic animal comprising

start site of bGH from the Pck_N promoter. Total liver RNA (10 μ g) was hybridized to a 280 bp 5' labelled probe from the Pck_NbGH gene in 40mM PIPES (Ph6.4), 1mM EDTA, 400mM NaCl, 80% formamide at 55° overnight. The probe spanned from the HinfI site in the 5' untranslated leader sequence of bGH to the PvuII site 5' of the TATA-17 box. The probe includes the tet-operator sequence of Pck_N (see Figure 3). After hybridization 300 μ l of ice cold digestion buffer (280mM NaCl, 50mM SODIUM ACETATE (Ph4.5), 4.5mM ZnSO₄, 20 μ g/ml carrier DNA and 500 units S1 nuclease) was added and incubated at 37° for 30 minutes. The reaction as stopped by adding 80 μ l of Stop Buffer (4M Ammonium acetate, 50mM EDTA and 50 μ g/ml tRNA), extracted with phenol/chloroform, precipitated with ethanol and analyzed on a 6% sequencing gel. The arrow indicates the protected fragment. Initiation of bGH mRNA from the modified Pck_N promoter occurs approximately 20 bp 3' of the TATA-box. This initiation site places the start of the message just prior to the first tetR binding site. This result indicates that the bGH mRNA starts from a single cap site, and suggests that tetR repression is due to a block in transcription initiation. Furthermore, unrepressed bGH expression appears to be due to limited tetR expression.

Figure 5. Nucleotide sequence of the tetR repressor protein gene.

Figure 6. Alternative, nonlimiting promoters of interest. Asterisks indicate sites at which tetR operator sequence may be inserted.

Figure 7. Northern blot analysis of bGH mRNA in liver

operator. Lower case letters correspond to polylinker sequence. The 5' EcoRI and 3' AccI restriction sites used for producing the modified PEPCK promoters (Pck_A and Pck-N) are indicated. The 10 base pair linker between OP1 and OP2 is underlined. Additional polylinker restriction sites available in p $\delta\delta 7$ include PstI, BamHI, SpeI, XbaI, NotI, EagI, SacII, BstXI, and SacI on the 5' side and XhoI, ApaI and KpnI on the 3' side.

Figure 2. A representation of the three modified PEPCK promoter elements. Construct 251 has the $\delta\delta 7$ operator sequence integrated in the AccI site of PEPCK, just 5' of the TATA-box control element. Construct 252 has the $\delta\delta 7$ operator sequence incorporated into the NheI site of PEPCK, just 3' of the TATA-box element. Construct 261 incorporates the TATA-specific operator sequence which is integrated between the 5' AccI site and the 3' NheI sites.

Figure 3. Structure of the modified PEPCK controlled bovine growth hormone genes. The Pck_AbGH and Pck_NbGH genes differ only in the site of operator insertion. For Pck_AbGH the operator is inserted at the AccI site 5' of the PEPCK TATA-box element. For Pck_NbGH the operator is inserted into the NheI site 3' of the TATA-box element (pPCK_NbGH has been deposited with the ATCC and assigned accession No:). In the Pck_TbGH gene, a TATA-box specific oligonucleotide was used, and this sequence was inserted between both the AccI and NheI sites.

A. Indicated the probe used for S1 hybridization.

Figure 4. S1 Nuclease protection assay to map the 5'

- 12 -

TZ-1: 5'CCGCATATGATCAATTCAAGGCCGAATAAG3'

TZ-3: 5'CTTTAGCGACTTGATGCTCTTGATCTTCCA3'

TZ-4: 5'AATTCGCCAGCCATGCCAAAAAAGAAGAGG3'

5 The TZ-4 primer is common to both primer pairs
and is the 5' primer which encompasses the start
codon of the tetR and mRNA. Primer TZ-1 and TZ-3
are two different 3' primers both of which are in
the tetR coding region. When amplified, these
10 primer pairs produced smaller than expected
products (approx. 215bp vs. 619bp for TZ-4 and
TZ-1, and approx. 94bp vs. 498bp for TZ-4 and
TZ-3). The products of this reaction were cloned
and sequenced. Sequencing revealed the presence
15 of an unexpected intron which spanned from near
the XbaI site at the start of tetR to a splice
acceptor just 8 base pairs 5' of the TZ-3 primer.

Figure 14. Composition analysis of Wild Type Tn10
tetR gene. The Tn10 tetR coding sequence was
20 analyzed on a desktop computer using Mac Vector
software. The figure shows a diagram of the tetR
coding region with the plus strand splice donor
(D) and splice acceptor (A) signal sequences
indicated. For reference the location of the
25 XbaI restriction is also indicated. The first
graph depicts the percentage of G and C bases in
the coding region of tetR. There are several
domains of very low GC content. The bottom graph
is an analysis of codon bias. The dark line is a
30 comparison of the tetR codon usage to a mouse
codon bias table. Values lower than 1.0 are
indicative of sequences which may translate
poorly. For reference, a comparison of tetR to a
Tobacco codon bias table is included (light
35 line). In transgenic tobacco, the tetR
regulation system functions very efficiently,

of F1 generation animals.

Figure 8. Northern blot analysis of bGH mRNA expression in four transgenic lines.

- 5 Figure 9A. Tissue specificity of bGH expression in Line 10-2 in the presence of 50 μ g/ml tetracycline. Northern blot analysis of bGH induction in a variety of tissues. Only the liver and kidney show significant expression.
- 10 Figure 9B. Tetracycline induction of bGH in Line 10-2. Both liver and kidney, which are the only sites for bGH expression in Figure 9A, also show tetracycline dependent bGH expression.
- Figure 10. 345 Repressor Construct.
- 15 Figure 11. Induction of bGH expression in Construct 345 Offspring. Northern blot analysis of liver RNA from F1 animals containing the 345 construct. Only animals from line 14 exhibit tetracycline dependent bGH expression.
- 20 Figure 12. Expression and alternative processing of tetR transgene. A RNase protection probe which extends from the NruI site of tetR 3' to the end of the gene was used. This probe includes only tetR coding sequences and should give a fully
- 25 protected fragment of approximately 400 base pairs. A protected fragment of approximately 220-260 base pairs is observed, which is far smaller than predicted.
- Figure 13. 5' Structure of tetR mRNA. Liver RNA was
- 30 treated with reverse transcriptase and amplified by PCR. The RNA was amplified using two different pairs of primers. The first primer pair (TZ-1 and TZ-4) should produce a 619 base pair product. The second primer pair (TZ03 and
- 35 TZ04) should produce a 498 base pair product. The sequence of the primers are:

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- (i) the tetR operator;
- (ii) modified promoters containing the tetR operator; and
- (iii) utility of the invention.

10

5.1. THE TETR OPERATOR

In order to practice the instant invention, the tetR operator sequence is inserted into a suitable animal promoter sequence in order to render that promoter subject to control by tetR repressor protein. A diagram of the tetR operator sequence is depicted in Figure 1.

It may be convenient to clone the tetR operator into a vector, such as a plasmid or a phage, to facilitate its propagation. Cloned operator sequence may then be rendered available for insertion into a promoter of interest, as set forth in Section 5.2., infra.

In a particular, nonlimiting embodiment of the invention, tetR operator sequence may be cloned as follows: Four oligonucleotides, which when annealed produce the two 19bp OP1 and OP2 palindromic sequences of the tetR operator may be synthesized; the sequences of said oligonucleotides are as follows:

- X-1. 5'ACTCTATCATTGATAGAGT3'
- X-2. 5'ACTCTATCAATGATAGAGT3'
- X-3. 5'TCCCTATCAGTGATAGAGA3'
- X-4. 5'TCTCTATCACTGATAGGGA3'

Oligonucleotides X-1 and X-2 are complementary and, when annealed, form the OP1 operator. Similarly, oligonucleotides X-3 and X-4, when annealed, produce

suggesting that for this gene, codon bias may be an important factor for efficient expression.

Figure 15. Synthetic tetR Component Sequences. The components of the synthetic tetR gene were synthesized by Midland Laboratories as four overlapping double stranded DNA cassettes. The sequence of these cassettes are shown. Each cassette was blunt cloned into the Hinc2 site of pUC19 and sequenced to verify authenticity. The resulting plasmids pLT1, pLT2, pLT3 and pLT5 can be used as the source material to assemble the entire synthetic tetR coding sequence since each contains an overlapping unique restriction site (bold face) through which they can be joined.

Figure 16. Sequence of Synthetic tetR gene.

Figure 17. Composition analysis of synthetic tetR.

These graphs were produced using the same software described in Figure 15. The figure depicts the structure of the synthetic tetR gene, now devoid of splice donor signal sequences, with only a single splice acceptor signal remaining (A). This is not the splice acceptor which was active in the 345 construct. The percentage of G and C bases has been significantly improved, while the frequency of CpG base pairs has been kept to a minimum. A CpG base pair is frequently the site for DNA methylation, which can negatively effect the expression of a gene. The codon bias of the synthetic tetR gene is also vastly improved. The graph depicts the results when the synthetic tetR coding sequence is compared to the same mouse codon bias table used previously.

For insertion of the operator sequence, the PEPCK promoter may be cut with NheI and end-filled with T4 polymerase; tetR operator, prepared as set forth in Section 5.1., supra, may then be blunt-ligated into place.

5.3. UTILITY OF THE INVENTION

5.3.1. STRATEGY

The strategy of the invention is to prepare a non-human transgenic animal that comprises two transgenes. The first transgene, termed "A," is a gene of interest, the expression of which is desirably controlled. Virtually any gene of interest may be used, including, but not limited to, growth hormone, hemoglobin, low density lipoprotein receptor, insulin, genes set forth in Table I, etc.

TABLE 1

Other Genes Of Interest

| Gene | Disease/Affect |
|-----------------------------------------------------|-------------------------|
| ADA Adenosine deaminase | Immuno-deficiency |
| TNF Tumor necrosis factor | Anti-cancer |
| IL-2 Interleukin-2 | Anti-cancer |
| LDL low density | hypercholesterolemia |
| Factor IX | hemophilia |
| Factor VIII | hemophilia |
| β -glucosidase | Gauchers disease |
| CFTR Cystic fibrosis | Cystic fibrosis |
| transmembrane regulator | |
| HPRT Hypoxanthine-guanine phosphoribosyltransferase | Lesch-Nyhan syndrome |
| UDP-glucuronyl transferase | Crigler-Najjar syndrome |
| Growth Hormone receptor | Growth |
| Insulin-like growth factor | Growth |
| Growth hormone releasing factor | Growth |

the OP2 operator site. The OP1 oligonucleotides may then be directly cloned into the EcoRV site of the Bluescript (Stratagene) polylinker to form plasmid X. OP2 oligonucleotides may then be cloned into a Mung bean nuclease blunted ClaI site of plasmid X to form plasmid Y. The resulting tetR operator may then be propagated and then excised from plasmid Y as an EcoRI, AccI fragment which may be end-filled with T4 polymerase and gel purified.

It is preferable that the separation between OP1 and OP2 is about 10-11 bp.

Analogous methods may be used to insert the tetR operator site into other suitable vectors.

5.2. MODIFIED PROMOTERS CONTAINING THE tetR OPERATOR

According to the invention, the tetR operator may be inserted into a suitable animal promoter so as to render that promoter subject to repression by tetR repressor protein. Any animal promoter maybe used; strategies for promoter selection are set forth in Section 5.3., infra.

In preferred embodiments of the invention, the tetR operator sequence is positioned 3' to the TATA-box sequence. A nonlimiting list of promoters which may be used according to the invention is set forth in Figure 6, together with the proximal portion of the promoter in the vicinity of the TATA-box, which is underlined.

In a specific, nonlimiting embodiment of the invention, the tetR operator site may be inserted into the NheI site of the PEPCK promoter (Wynshaw-Boris et al., 1984, J. Biol. Chem. 259:12161-12169). A diagram of the PEPCK promoter containing the tetR operator sequence of the NheI site is presented in Figure 2.

_____, _____, _____, and _____, as described in Section 7, infra.

5 In further embodiments, the present invention provides for additional synthetic tetR genes from which one or more splice sites have been deleted or for which codon usage has been further optimized.

10 The present invention covers synthetic tetR genes having the sequence set forth in Figure 16 and for functionally equivalent variants of that sequence.

In specific, non-limiting embodiments of the invention, a nuclear localization signal may be added to a natural or synthetic tetR gene to facilitate its expression (See, Section 7, infra).

15 Expression of tetR is controlled by promoter "C". While it is preferable that promoter C be the same as promoter B except that promoter C does not contain a tetR operator sequence, any promoter which provides expression of tetR so as to repress expression of gene
20 "A" during the period when it is desirable to repress expression of "A" may be used.

For example, and not by way of limitation, a transgenic animal may be produced which carries a first transgene which is bovine growth hormone under
25 the control of a PEPCK promoter modified to contain a tetR operator sequence at the NheI site and a second transgene which is tetR repressor protein under the control of an unmodified PEPCK promoter; see Section 6, infra. The pPCK_NbGH construct has been deposited
30 with the ATCC and assigned accession number _____.

5.3.2. TRANSGENIC ANIMALS OF THE INVENTION

35 The binary repressor system of the invention may be used to control gene expression in any non-human transgenic animal, including, but not limited to, transgenic mice, pigs, goats, cows, rabbits, sheep,

The expression of gene "A" is under the transcriptional control of promoter "B". Promoter B comprises a tetR operator sequence, as discussed supra. Promoter B desirably defines the time and tissue window in which the transgene may be induced; for example, promoter A may be a tissue specific promoter such as the PEPCK promoter (which is expressed selectively in liver and becomes active shortly prior to birth). The second transgene encodes the tetR repressor, the sequence of which is set forth in Figure 5.

Analysis of the Tn10 tetR coding sequence indicates that the codon usage for this gene is poorly suited for expression in mammalian cells (FIG. 15). To optimize tetR expression in mammalian cells a new tetR repressor gene was designed (See, Section 7, infra), which may be utilized in alternative embodiments of the invention. The synthetic tetR gene (syn-tetR) is designed to encode exactly the same protein product as the bacterial Tn10 tetR gene but optimizes codon usage for mammalian cells. The percentage of G and C bases has been significantly improved, while the frequency of CpG base pairs has been minimized. A CpG base pair is frequently the site for DNA methylation which can negatively affect the expression of a gene. In addition, the syn-tetR gene is devoid of any splice signals, decreasing the likelihood of aberrant splicing of the RNA which may result in production of a non-functional message. The sequence of the synthetic tetR gene is depicted in Figure 16. Plasmids comprising these sequences may be constructed using plasmids pLT-1, pLT-2, pLT-3 and pLT-5 (deposited with the American Type, Culture Collection (ATCC) and assigned accession numbers

for 12 to 14 days. On the last day of AT feeding all gilts may be given an intramuscular injection of prostaglandin F_{2α} (Lutalyse: 10mg/injection) at 0800 and 1600 hours. Twenty-four hours after the last day of AT consumption all donor gilts may be administered a single intramuscular injection of pregnant mare serum gonadotrophin (1500 U). Human chorionic gonadotrophin (750 IU) may be administered to all donors at 80 hours after pregnant mare serum gonadotrophin.

Following AT withdrawal, donor and recipient gilts may be checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus within 36 hours following human chorionic gonadotrophin administration may be bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG one- and two-cell ova may be surgically recovered from bred donors using the following procedure. General anesthesia may be induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg of ketamine/kg via a peripheral ear vein. Following anesthetization, the reproductive tract may be exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) may be inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova may then be flushed in retrograde fashion by inserting a 20g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) may be infused into the oviduct and flushed toward the glass cannula. The medium may be collected into

etc. The present invention provides for such non-human transgenic animals carrying as transgenes nucleic acid constructs described herein, including natural or synthetic tetR repressor proteins and operator sequences.

Transgenes may be introduced by microinjection, transfection, transduction, electroporation, cell gun, embryonic stem cell fusion, or any other method known in the art. The transgenes of the invention may be co-introduced into a single animal or may be introduced into two individual animals that are subsequently mated to produce doubly transgenic offspring.

For example, for the production of transgenic mice, the following general protocol may be used. Male and female mice are mated at midnight. Twelve hours later, the female may be sacrificed and the fertilized eggs may be removed from the uterine tubes. Foreign DNA may then be microinjected (100-1000 molecules per egg) into a pronucleus. Shortly thereafter, fusion of the pronuclei (a pronucleus or the male pronucleus) occurs, and, in some cases, foreign DNA inserts into (usually) one chromosome of the fertilized egg or zygote. The zygote may then be implanted into a pseudo-pregnant female mouse (previously mated with a vasectomized male) where the embryo develops for the full gestation period of 20-21 days. The surrogate mother then delivers the mice and by four weeks transgenic pups may be weaned from the mother.

According to another embodiment of the invention, a transgenic pig may be produced, briefly, as follows. Estrus may be synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (e.g. allyl trenbolone, AT: 15mg/gilt/day)

mls of BMOC-3 medium may be aspirated into the tubing. The tubing may then be fed through the ostium of the oviduct until the tip reaches the lower third or
5 isthmus of the oviduct. The ova may be subsequently expelled as the tubing is slowly withdrawn. The exposed portion of the reproductive tract may be bathed in a sterile 10% glycerol - 0.9% saline solution and returned to the body cavity. The
10 connective tissue encompassing the linea alba, the fat, and the skin may be sutured as three separate layers. An uninterrupted Halstead stitch may be used to close the linea alba. The fat and skin may be closed using a simple continuous and mattress stitch,
15 respectively. A topical antibacterial agent (e.g. Furazolidone) may then be administered to the incision area. Recipients may be penned in groups of about four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18
20 (day 0 = onset of estrus), all recipients may be checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection may be performed using ultrasound. On day 107 of gestation recipients may be transferred to the farrowing suite. In order to
25 ensure attendance at farrowing time, farrowing may be induced by the administration of prostaglandin F₂ (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients may be expected to farrow with 34 hours following PGF 2a
30 administration.

As used herein, the term "transgenic animal" refers to animals that carry a transgene in at least some of their somatic cells, and preferably in at least some of their germ cells.

35

- 21 -

sterile 17 x 100 mm polystyrene tubes. Flushings may be transferred to 10 x 60 mm petri dishes and searched at a lower power (50x) using a Wild M3

5 stereomicroscope. All one- and two- cell ova may be washed twice in Brinster's Modified Ova Culture -3 medium (BMOC -3) supplemented with 1.5% BSA and transferred to 50 μ l drops of BMOC-3 medium under oil. Ova may be stored at 38°C under a 90% N₂, 5% O₂, 5% CO₂

10 atmosphere until microinjection is performed. One and two-cell ova may be placed in an Eppendorf tube (15 ova per tube) containing 1 ml HEPES medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14,000g in order to visualize pronuclei in

15 one-cell and nuclei in two-cell ova. Ova may then be transferred to a 5-10 μ l drop of HEPES medium under oil on a depression slide. Microinjection may be performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700

20 molecules of construct DNA (linearized at a concentration of about 1ng/ μ l of Tris-EDTA buffer) may be injected into one pronucleus in one-cell ova or both nuclei in two-cell ova. Microinjected ova may be returned to microdrops of BMOC-3 medium under oil and

25 maintained at 38°C under a 90% N₂, 5% CO₂, 5% O₂ atmosphere prior to their transfer to suitable recipients. Ova may preferably be transferred within 10 hours of recovery. Only recipients which exhibit estrus on the same day or 24 hours later than the

30 donors may preferably be utilized for embryo transfer. Recipients may be anesthetized as described supra. Following exteriorization of one oviduct, at least 30 injected one- and/or two-cell ova and 4-6 control ova may be transferred in the following manner. The

35 tubing from a 21g x 3/4 butterfly infusion set may be connected to a lcc syringe. The ova and one to two

tetracycline, as inducer, is between about 5-50 mg/kg and preferably between about 5-15 mg/kg.

5 6. EXAMPLE: TETRACYCLINE REPRESSOR-MEDIATED
BINARY REGULATION SYSTEM FOR CONTROL OF
BOVINE GROWTH HORMONE EXPRESSION IN
TRANSGENIC MICE

 6.1. MATERIALS AND METHODS

 6.1.1. CONSTRUCTION OF PLASMIDS

10 Plasmid p $\delta\delta 7$ contains a functional tetR operator
site cloned within a Bluescript (Stratagene)
polylinker. This plasmid is useful for propagating
the operator sequence, and as a source of operator
sites for insertion into the PEPCK promoter or any
15 other promoter element. The p $\delta\delta 7$ plasmid was made as
follows. Four oligonucleotides, which when annealed
produce the two 19bp OP1 and OP2 palindromic sequences
of the tetR operator were synthesized. The sequences
of each oligonucleotide is listed below.

20 X-1.5' ACTCTATCATTGATAGAGT 3'
 X-2.5' ACTCTATCAATGATAGAGT 3'
 X-3.5' TCCCTATCAGTGATAGAGA 3'
 X-4.5' TCTCTATCACTGATAGGGA 3'

 Oligonucleotides X-1 and X-2 are complementary and
25 when annealed form the OP1 operator. Similarly
oligonucleotides X-3 and X-4 produce the OP2 operator
site. The OP1 oligonucleotides were directly cloned
into the EcoRV site of the Bluescript polylinker. The
resulting plasmid pSOPI was sequenced to verify the
30 integrity of the insert. OP2 oligonucleotides were
subsequently cloned into a Mung bean nuclease blunted
Clal site of pSOPI to produce p $\delta\delta 7$. Due to a cloning
artifact produced by the Mung bean nuclease, the
operator in p $\delta\delta 7$ consists of the two 19bp OP1 and OP2
35 sequences separated by linker of only 10 base pairs.
This difference does not effect tetR binding. The

5.3.3. INDUCTION

Induction of expression of the gene of interest in transgenic animals of the invention may be achieved by administering, to the animal, a compound that binds to tetR so that tetR repressor function is inhibited. Examples of such compounds include tetracycline and tetracycline-like compounds, including, but not limited to, apicycline, chlortetracycline, clomocycline, demeclocycline, guamecycline, lymecycline, meclocycline, methacycline, minocycline, oxytetracycline, penimepicycline, pipacycline, rolitetracycline, sancycline, and senociclin.

Administration of the inducer can be through direct injection, water, feed, aerosol, or topical application. The choice of method will depend on the promoters used and the specific application of the transgenic animals. For example, injection, water and feed would provide inducer to all of the animals tissues. In our case, administration through water or feed would be the preferred method to control growth hormone expression in transgenic pigs. Aerosol spray could be used to attain high antibiotic concentrations in the lung. This may be appropriate for example in a cystic fibrosis or emphysema model. Topical application to the skin is also possible and could be used in models of acne, hair loss, wound healing or viral infection.

Induction of the gene of interest is accomplished by administering an effective amount of inducer, as described above. An effective amount of inducer may be construed to mean that amount which increases expression of the gene of interest by at least about 50 percent. As the LD₅₀ for tetracycline HCl in rats is about 6643 mg/kg and the therapeutic dose is between about 25-50 mg/kg, an effective dose of

untranslated DNA. This insert was excised from the parent plasmid and subcloned into a plasmid with a more suitable polylinker to produce pSTET7. To this
5 plasmid a 870bp XhoI, BamHI fragment derived from pMSG (Pharmacia), containing the SV40 small-T intron and polyadenylation signal sequences was inserted at the HindII site 3' of the tetR coding region to produce pSTetRSv. Finally an unmodified 610bp PEPCK promoter
10 was inserted at the EcoRI site of pSTetRSv to produce pPck_tetRSv. The PEPCK promoter is identical to the promoter used to produce pPck_A, pPck_N, and pPck_T except that it does not contain a tetR operator site. This PEPCK promoter has been previously used in
15 transgenic animals and is known to target gene expression specifically to the liver.

6.1.3. GROWTH HORMONE GENES

Plasmid pGH-SAF107 contains a 2.2kb BamHI, EcoRI
20 genomic fragment of the bovine growth hormone (bGH) gene, blunt ligated into an EcoRV site. To this vector each of the modified PEPCK promoters was added by blunt ligating the promoter into the BamHI site of pGH-SAF107. The structure of the resulting plasmids
25 is depicted in Figure 3. Plasmid pPCK_NbGH was deposited with the ATCC and assigned accession number _____. For production of transgenic animals, each of the PEPCK-bGH genes was excised from the vector using XhoI and SacI, gel fractionated and
30 purified using an Elutip column.

6.1.4. TRANSGENIC MICE

Transgenic mice were made which contain both the Pck_tetRSv gene and one of the modified PEPCK
35 promoters controlling bGH. Table 2 lists the number

sequence of the p $\delta\delta 7$ operator site is shown in Figure 1B. The 55 base pair tetR operator was excised from p $\delta\delta 7$ as an EcoRI, AccI fragment, end filled with T4
5 polymerase, and gel purified. This fragment was subsequently used to produce the modified PEPCK promoters Pck_N and Pck_A.

Plasmids Pck_A and Pck_N were produced by inserting the 55bp tetR operator into the unique AccI
10 and NheI sites (respectively) of the PEPCK promoter (pPCK_NbGH has been deposited with ATTC and assigned accession No:). For both plasmids the promoter was cut with the appropriate restriction enzyme, end
filled with T4 polymerase and the tetR operator blunt
15 ligated into place. A third modified PEPCK promoter, Pck_T was produced in which the OP1 and OP2 operator sequences were positioned to flank the PEPCK TATA-box element. To produce Pck_T a new oligonucleotide
(5'ACTCTATCATTGATAGAGTTACTAT
20 TTAAATCCCTATCAGTGATAGAGA3') was produced. This oligonucleotide was kinased with T4 polynucleotide kinase and annealed to kinased X-2 and X-4 which are complementary to the first and last 19bp. The complete double stranded 49bp operator was produced by
25 filling in the 11bp linker region, which includes the PEPCK TATA-box element, with Klenow. The final product was then blunt cloned into an AccI, NheI cut PEPCK promoter. All three modified promoters were sequenced to verify the inserts. Figure 2 depicts the
30 structure of these promoters.

6.1.2. REPRESSOR CONSTRUCT

Plasmid pBI501 contains a 701 bp HincII fragment from E. coli Tn10, cloned into the HincII site of
35 pUC8. The HincII insert contains the entire tetR coding sequence along with 21bp of 5' and 55bp of 3'

At 10 weeks of age, a sampling of transgenic female founders containing the A+T and N+T were tested for induction of bGH in the serum using a radio-immune
 5 assay, after a single IP injection of 60 mg/kg tetracycline-HCl. The purpose of this experiment was simply to determine which if either of these two modified promoters was responsive to repression by tetR. The results are summarized in Table 4.

TABLE 4

| Construct | Animal | Weight | Basal | 12 hours | 36 hours |
|-----------|-------------|--------|------------------|------------------|------------------|
| 249 | 2-5 female | 21.1 | 0.00 | 0.00 | 0.00 |
| 250 | 6-6 female | 42.9 | 4.6 \pm 0.033 | 3.4 \pm 0.062 | 4.9 \pm 0.072 |
| 251 | 6-6 female | 19.3 | 0.00 | 0.00 | 0.00 |
| 251 | 10-5 female | 25.1 | 0.20 \pm 0.008 | 0.19 \pm 0.001 | 0.21 \pm 0.038 |
| 252 | 5-2 female | 38.7 | 0.59 \pm 0.107 | 0.64 \pm 0.044 | 1.12 \pm 0.207 |
| 252 | 5-3 female | 20.0 | 0.00 | 0.00 | 0.00 |
| 252 | 10-2 | 19.2 | 0.00 | 0.00 | 0.00 |

No induction of bGH was observed in animals that lack the Pck_tetRSV gene (construct 250) or in animals with
 25 both the Pck_AbGH + Pck-tetRSV genes (construct 251). An approximate two fold increase in serum bGH levels was however detected in the 5-2 female which contains the Pck-NbGH + Pck_tetRSV genes. The remainder of the animals had undetectable levels of bGH expression, due
 30 in part to the relatively low sensitivity of this assay. For example the 10-2 female (construct 252) shows no detectable bGH in the serum, but subsequent experiments on her offspring indicate that this line of animals does express bGH mRNA in a tetracycline
 35 dependent manner. This initial data, suggested that

of eggs injected, offspring produced and number of transgenics derived for each construct.

TABLE 2

| Construct | Eggs injected | Eggs transferred | Live Born | Transgenic |
|--------------------------------|---------------|------------------|-----------|------------|
| Pck_AbGH + Pck_tetRSv (251) | 233 | 194 | 40 | 14 (0.35) |
| Pck-NbGH + Pck_tetRSv (252) | 268 | 208 | 30 | 9(0.3) |
| Pck_TbGH + Pck-tetRSv (261) | 227 | 197 | 25 | 5(0.2) |

6.2. RESULTS AND DISCUSSION

15 Once the transgenic founder animals were identified, they were weighed each week. Table 3 lists the mean weights of each group of transgenic animal at 11 weeks of age.

TABLE 3

| Construct | Sex | Weight |
|--------------------------|--------|----------------|
| Pck_AbGH + Pck_tetRSv(9) | male | 36.122(12.251) |
| Pck_AbGH + Pck_tetRSv(4) | female | 29.125(7.861) |
| Pck_NbGH + Pck_tetRSv(5) | male | 34.840(14.745) |
| Pck_NbGH + Pck_tetRSv(4) | female | 28.125(10.958) |
| Pck_TbGH + Pck_tetRSv(3) | male | 36.267(11.402) |
| Pck_TbGH + Pck_tetRSv(2) | female | 27.300(5.798) |
| NON-TRANSGENIC(6) | male | 29.583(2.395) |
| NON-TRANSGENIC(6) | female | 23.117(1.863) |

35 As expected for each co-injection, large animals, obviously expressing elevated levels of bGH, were observed as were animals of normal stature.

An S1 nuclease protection assay was performed to identify the start site of transcription of bGH mRNA. As shown in Figure 4, there was only one start site identified regardless of the presence or absence of tetR repressor binding. This start site was located approximately 20 bp downstream from the TATA-box. At this location, the message is initiating within the 007 operator sequence, just 3 or 4 base pairs 5' of the first tetR binding site.

7. EXAMPLE: OPTIMIZATION OF tetR CODING SEQUENCE

The use of the wild type Tn10 tetR gene in conjunction with the 252 construct indicates that the TetR system can function in transgenic animals and that in some cases, for instance in the 10-2 transgenic animals, the level of regulation can be very high (FIGS. 9A and 9B). However, in other instances the efficiency of repression is not always complete, leading to a significant basal level of bGH expression. This failure to repress may be due to low level expression of tetR. To optimize the expression of tetR repressor, a synthetic tetR gene was generated which was devoid of splice signals and had optimized codon usage for mammalian cells.

7.1 MATERIALS AND METHODS

7.1.1. TISSUE SPECIFICITY AND TETRACYCLINE INTRODUCTION OF bGH IN LINE 10-2

For all Northern blots 10µg of whole RNA was electrophoreses through a 1% agarose gel containing 3% formaldehyde using standard techniques. To detect bGH mRNA a random primed, radioactive bGH cDNA probe was used. All conditions for hybridization and washing of

the Pck_N promoter was being regulated by tetR at least to a limited extent.

To further characterize the mice, improve the
5 sensitivity of the assay and to test the
responsiveness of the Pck_T promoter, offspring of
founder mice from each co-injection were produced.
The transgenic progeny were then raised in the
presence or absence of tetracycline medicated water
10 (500µg/ml) for 4 weeks, prior to analysis of bGH mRNA
expression levels in the liver, the predominant site
of PEPCCK expression. Northern blot hybridization
analysis of these animals (Figure 7) demonstrated
again, that animals with the Pck_NbGH gene were
15 responsive to repression by tetR and that the other
two modified promoters exhibited no signs of tetR
dependent regulation.

We attempted to breed all of the remaining
founders containing the Pck-NgGH + Pck_tetRSv genes to
20 analyze their offspring in a similar manner (Figure
8). Of the 5 founders which produced offspring, 2 did
not express bGH under any conditions, and from the
remaining 3 one segregated two different integration
sites allowing us to establish a total of 4 lines.
25 All 4 lines exhibited tetracycline dependent bGH
expression as assayed by Northern blot hybridization.
The efficiency of tetR repression appeared to be
inversely correlated with the level of expression.
For example 9-5 animals have the highest level of bGH
30 expression, show an obvious increase in body size, and
exhibit only marginal tetR repression. In contrast 9-
4Lc and 10-2 animals exhibit lower levels of
tetracycline induced bGH expression, are of normal
stature and appear to be efficiently regulated by
35 tetR.

produce smaller than expected products (approx. 215bp vs. 619bp for TZ-4 and TZ-1, and approx. 94bp vs. 498bp for TZ-4 and TZ-3). The products of this
5 reaction were cloned and sequenced. The sequence revealed the presence of an unexpected intron which spanned from near the XbaI site at the start of tetR to a splice acceptor just 8 base pairs 5' of the TZ-3 primer.

10

7.1.4. 345 REPRESSOR CONSTRUCT

In an embodiment of the invention, any nuclear localization signal may be added to a natural or synthetic tetR gene to facilitate its expression. For
15 example, complementary oligonucleotides which encode a nuclear localization signal sequence were synthesized (Oligos etc.) and added in frame to the tetR coding sequences of pSTETR107 at the EcoRI and XbaI restriction sites to produce pNTETR. Oligonucleotide
20 sequences are:

(GB1) 5'AATTCGCCAGCCATGCCAAAAAGAAGAGGAAGGTAT3' and
(GB2) 5'CTAGATACCTTCCTCTTCTTTTTGGCATGGCTGGC3'.

When annealed these oligonucleotides have a 5' EcoRI and 3' XbaI compatible overhangs. These
25 oligonucleotides fuse the amino acid sequence Met Pro Lys Lys Lys Arg, Lys Val, to the third amino acid (Arg) of wild type tetR.

Two complementary 51 base pair oligonucleotides which start the 5' cap site of bGH and extend to the
30 first exon were synthesized (Oligos etc.). Sequence for the oligonucleotides are (5b-1):

5'GATCCCAGGACCCAGTTCACCAGACGACTCAGGGTCCTGTGGACAGCT
CAG3'

and (5b-2):

35 5'AATTCTGAGCTGTCCACAGGACCCTGAGTCGTCTGGTGAAGTGGGTCC

filters were done in accordance with standard techniques of molecular biology.

5 7.1.2. EXPRESSION AND ALTERNATIVE PROCESSING
 OF THE tetR TRANSGENE

 A RNase protection probe which extended from the
NruI site of tetR 3' to the end of the gene was used.
This probe includes only tetR coding sequences and
10 should give a fully protected fragment of
approximately 400 base pair. When hybridized to 150µg
of liver RNA (500,000 cpm of probe in a 30µl
hybridization consisting of 80% formamide, 40mM PIPES
pH 6.4, 400mM NaOAc, and 1mM EDTA), and digested with
15 RNase one (Promega) for 30 minutes at 37° as
recommended by the manufacturer, a protected fragment
of approximately 221-260 base pairs is observed, far
smaller than predicted.

20 7.1.3. 5' STRUCTURE OF tetR mRNA

 Liver RNA was treated with reverse transcriptase
and amplified by PCR using the manufacturers
recommended conditions (Pharmacia). The RNA was
amplified using two different pairs of primers. The
25 first primer pair (TZ-1 and TZ-4) should produce a 619
base pair product. The second primer pair (TZ-3 and
TZ-4) should produce a 498 base pair product. The
sequence of the primers are:

TZ-1: 5'CCGCATATGATCAATTCAAGGCCGAATAAG3'
30 TZ-3: 5'CTTTAGCGACTTGATGCTCTTGATCTTCCA3'
TZ-4: 5'AATTCGCCAGCCATGCCAAAAAGAAGAGG3'

 The TZ-4 primer is common to both primer pairs
and is the 5' primer which encompasses the start codon
of the tetR mRNA. Primer TZ-1 and TZ-3 are two
35 different 3' primers both of which are in the tetR
coding region. When amplified, these primer pairs

Sa11 and Sac1. This fragment was gel purified and coinjected with the PCK-NbGH gene previously described to generate transgenic mice.

5

7.1.5. SYNTHETIC tetR COMPONENT SEQUENCES

The components of the synthetic tetR gene were synthesized by Midland Laboratories as four overlapping double stranded DNA cassettes. The sequence of these cassettes are shown in Figure 15. Each cassette was blunt cloned into the Hinc2 site of pUC19 and sequenced to verify authenticity. The resulting plasmids pLT1, pLT2, pLT3 and pLT5 can be used as the source material to assemble the entire synthetic tetR coding sequence since each contains an overlapping unique restriction site (bold face) through which they can be joined (pLT-1, pLT-2, pLT-3 and pLT-5 have been deposited with ATCC and have been assigned accession numbers ___, ___, ___, and ___ respectively). There are many possible ways by which these cassettes can be joined. By way of an example, the inserts of plasmid pLT1 and pLT2 can be excised using EcoR1 and Nsi1. The inserts can then be combined by cloning these two fragments into an EcoR1 vector. This procedure will assemble the 5' half of the gene, using the overlapping Nsi1 restriction site to join the pieces. Similarly, the 3' half of the gene can be assembled from pLT3 and pLT5 by cutting with EcoR1 and Sph1 (pLT3) and Sph1 and Hind III (pLT5) to release the inserts. These inserts can then be joined at the overlapping Sph1 site by cloning the fragments into an EcoR1, Hind III cut vector. Finally, the entire coding region can be put together using the overlapping restriction site Apa1. This would result in a vector with the synthetic tetR

35

TGG3'. When annealed these oligonucleotides have 5' BamH1 and 3'EcoR1 compatible overhands. The oligonucleotides for the 5' leader sequence of bGH were cloned into a BamH1, EcoR1 cut plasmid to produce p5'GH.

The nuclear localization modified tetR coding sequence was isolated by gel purification after restriction digestion of pNTETR using EcoR1 and Hind III. This fragment was then inserted into p5'GH at the EcoR1 and Hind III sites to product p5'GHTR.

To add the remainder of the bGH genomic sequence an intermediate modification of p5'GHTR was first made. This modification consisted of adding a Hind III - Pst1 linker to the Hind III site of p5'GHTR to product pGT0. The sequence of the oligonucleotides which comprise this linker are: (CC-1) 5'AGCTTCTGCAG3' and (CC-2) 5'AGCTCTGCAGA3'. The remaining bGH genomic sequences were added in two steps. First the Pst1 Sac2 fragment that begins in the first exon of bGH and ends in the third intron was excised from pSGH107. Similarly, the insert of pGT0 which contains the 5' untranslated leader of bGH and the nuclear localization modified tetR was excised using BamH1 and Pst1. These two gel purified fragments was then cloned into a BamH1 Sac2 cut vector to produce pGTG. Finally, the remainder of the bGH gene from the Sac2 site in the third intron to the end of the gene, was added to pGTG by cutting pGTG with Sac2 and adding the Sac2 fragment from pSGH106 to produce pNTETR-GH.

Plasmid pNTETR-GH was digested with BamH1 to excise the NTETR-GH gene. The fragment was cloned into the BamH1 site of pPCK 305 to produce the final plasmid pPCK-GHNTET. To produce transgenic mice, the PEPCK-GHTET gene was excised from the plasmid using

tetR gene is vastly improved. The graph depicts the results when the synthetic tetR coding sequence is compared to the same mouse codon bias table used previously.

7.2 RESULTS

7.2.1. EXPRESSION OF tetR IN CONSTRUCT 345 OFFSPRING

To improve tetR expression a new repressor construct was produced. The construct, referred to as Construct 345 is depicted in Figure 10. In the 345 construct the coding region of tetR is augmented with a nuclear localization signal sequence to increase the nuclear concentration of repressor. The tetR coding region was inserted into the first exon of the bGH gene. The bGH gene then acts as a genomic carrier, providing multiple introns, which may improve expression, and a strong polyadenylation signal, which may improve the processing and stability of the message.

The new repressor was coinjected with the bGH gene from construct 252. The resulting transgenic animals contain the new repressor, and a PEPCK regulated bGH gene with the tetR operators located just 3' of the PEPCK TATA-box element. Offspring of these animals were screened for bGH induction (FIG. 11). Of the lines tested only one, line 14, showed tetracycline dependent regulation of bGH, and in this one case there was still a significant base level of bGH expression. Northern analysis, performed to determine the levels of tetR mRNA expressed in the transgenic mice, indicated that the tetR gene was still not expressed at a high level.

To detect tetR mRNA with higher sensitivity the tetR mRNA was analyzed using RNase protection. This technique revealed that the mRNA was shorter than

coding sequence, as depicted in Figure 16, cloned into a plasmid as an EcoR1 Hind III fragment.

5 7.1.6. COMPOSITIONAL ANALYSIS OF
 WILD TYPE Tn10 tetR GENE

10 The Tn10 tetR coding sequence was analyzed on a
desktop computer using Mac Vector software. Figure 14
shows a diagram of the tetR coding region with all of
the plus strand splice doner (D) and splice acceptor
15 (A) signal sequences indicated. For reference the
location of the XbaI restriction is also indicated.
The first graph depicts the percentage of G and C
bases in the coding region of tetR. There are several
domains of very low GC content. The bottom graph is
15 an analysis of codon bias. The dark line is a
comparison of the tetR codon usage to a mouse codon
bias table. Values much lower than 1.0 are indicative
of sequences which may translate poorly. For
20 reference, a comparison of tetR to a Tobacco codon
bias table is included (light line). In transgenic
tobacco, the tetR regulation system functions very
efficiently, suggesting that for this gene, codon bias
may be an important factor for efficient expression.

25 7.1.7. COMPOSITIONAL ANALYSIS OF SYNTHETIC tetR

Figure 17 depicts the structure of the synthetic
tetR gene, now devoid of splice donor signal
sequences, with only a single splice acceptor signal
30 remaining (A). This is not the splice acceptor which
was active in the 345 construct. The percentage of G
and C bases has been significantly improved, while the
frequency of CpG base pairs has been kept to a
minimum. A CpG base pair is frequently the site for
35 DNA methylation, which can negatively effect the
expression of a gene. The codon bias of the synthetic

greatly improved codon usage for mammalian cells. The sequence of the of the syn-tetR is indicated in Figure 16. The predicted analysis for splicing signals, G+C content, and codon usage are depicted in Figure 17.

8. DEPOSIT OF MICROORGANISMS

The following microorganisms have been deposited with the American Type Culture Collection, (ATCC), Rockville, Maryland and have been assigned the following accession numbers:

| | <u>Microorganism</u> | <u>Date of Deposit</u> | <u>Accession No.</u> |
|----|----------------------|------------------------|----------------------|
| | pLT-1 | August 25, 1993 | |
| | pLT-2 | August 25, 1993 | |
| 15 | pLT-3 | August 25, 1993 | |
| | pLT-5 | August 25, 1993 | |
| | pPCK_NbGH | August 25, 1993 | |

The present invention is not to be limited in scope by the microorganisms deposited since the deposited embodiments are intended as illustrations of single aspects of the invention and any microorganisms which are functionally equivalent are within the scope of the invention.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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expected (FIG. 12). Subsequent analysis using reverse transcriptase-PCR with primers that amplify the entire coding region of tetR confirmed that the mRNA was significantly shorter than expected (FIG. 13). Sequence analysis of these RT-PCR products indicated that an unexpected splicing event had occurred. This splicing process occurred between a splice donor signal in the 5' end of the tetR coding region and a splice acceptor approximately 400 bp 3' of the start codon. The resulting mRNA is therefore deleted of the tetR DNA binding domain and about two third of the entire coding region. This mRNA could not possibly make a functional repressor.

15

7.2.2. OPTIMIZATION OF tetR CONSTRUCT

A more detailed analysis of the tetR coding sequence indicated that the codons used in this gene are poorly suited for expression in mammalian cells (FIG. 14). Therefore, it appears that the inefficiency of the tetR system is the result of two processes: (i) aberrant splicing of the RNA to produce a nonfunctional message; and (ii) inefficient translation which can lead to rapid mRNA turnover.

To circumvent the problems of internal splicing and potential problems due to codon bias and G-C content, a synthetic tetR gene was designed. The components of the synthetic tetR gene were synthesized as four overlapping double stranded cassettes. Each cassette was cloned in puc19. The resulting plasmids designated pLT-1, pLT-2, pLT-3 and pLT-5, as depicted in Figure 15, have been deposited with ATCC and assigned accession numbers _____, _____, _____, and _____, respectively. The synthetic tetR (syn-tetR) has been designed to encode exactly the same protein product, but is devoid of splice signals and has

- 40 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Byrne, Guerard
- (ii) TITLE OF INVENTION: TETRACYCLINE REPRESSOR-MEDIATED BINARY REGULATION SYSTEM FOR CONTROL OF GENE EXPRESSION IN TRANSGENIC ANIMALS
- (iii) NUMBER OF SEQUENCES: 15
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Pennie & Edmonds
 - (B) STREET: 1155 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 10036-2711
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/935,763
 - (B) FILING DATE: 26-AUG-1992
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Coruzzi, Laura A.
 - (B) REGISTRATION NUMBER: 30,742
 - (C) REFERENCE/DOCKET NUMBER: 6794-025
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 212 790-9090
 - (B) TELEFAX: 212 869-8864/9741
 - (C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 59 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGACACTCT ATCATTGATA GAGTTATTTT ACCACTCCCT ATCAGTGATA GAGAAAAGT

59

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 70 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

5 Various publications are cited herein, which are hereby incorporated by reference in their entirety.

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- 42 -

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| Leu | Asn | Glu | Val | Gly | Ile | Glu | Gly | Leu | Thr | Thr | Arg | Lys | Leu | Ala | Gln | | |
| | | | 20 | | | | | 25 | | | | | 30 | | | | |
| AAG | CTA | GGT | GTA | GAG | CAG | CCT | ACA | TTG | TAT | TGG | CAT | GTA | AAA | AAT | AAG | 144 | |
| Lys | Leu | Gly | Val | Glu | Gln | Pro | Thr | Leu | Tyr | Trp | His | Val | Lys | Asn | Lys | | |
| | | 35 | | | | | 40 | | | | | 45 | | | | | |
| CGG | GCT | TTG | CTC | GAC | GCC | TTA | GCC | ATT | GAG | ATG | TTA | GAT | AGG | CAC | CAT | 192 | |
| Arg | Ala | Leu | Leu | Asp | Ala | Leu | Ala | Ile | Glu | Met | Leu | Asp | Arg | His | His | | |
| | | 50 | | | | 55 | | | | | 60 | | | | | | |
| ACT | CAC | TTT | TGC | CCT | TTA | GAA | GGG | GAA | AGC | TGG | CAA | GAT | TTT | TTA | CGT | 240 | |
| Thr | His | Phe | Cys | Pro | Leu | Glu | Gly | Glu | Ser | Trp | Gln | Asp | Phe | Leu | Arg | | |
| | | 65 | | | 70 | | | | 75 | | | | | | 80 | | |
| AAT | AAC | GCT | AAA | AGT | TTT | AGA | TGT | GCT | TTA | CTA | AGT | CAT | CGC | GAT | GGA | 288 | |
| Asn | Asn | Ala | Lys | Ser | Phe | Arg | Cys | Ala | Leu | Leu | Ser | His | Arg | Asp | Gly | | |
| | | | | 85 | | | | | 90 | | | | | 95 | | | |
| GCA | AAA | GTA | CAT | TTA | GGT | ACA | CGG | CCT | ACA | GAA | AAA | CAG | TAT | GAA | ACT | 336 | |
| Ala | Lys | Val | His | Leu | Gly | Thr | Arg | Pro | Thr | Glu | Lys | Gln | Tyr | Glu | Thr | | |
| | | | 100 | | | | | 105 | | | | | 110 | | | | |
| CTC | GAA | AAT | CAA | TTA | GCC | TTT | TTA | TGC | CAA | CAA | GGT | TTT | TCA | CTA | GAG | 384 | |
| Leu | Glu | Asn | Gln | Leu | Ala | Phe | Leu | Cys | Gln | Gln | Gly | Phe | Ser | Leu | Glu | | |
| | | 115 | | | | 120 | | | | | | 125 | | | | | |
| AAT | GCA | TTA | TAT | GCA | CTC | AGC | GCT | GTG | GGG | CAT | TTT | ACT | TTA | GGT | TGC | 432 | |
| Asn | Ala | Leu | Tyr | Ala | Leu | Ser | Ala | Val | Gly | His | Phe | Thr | Leu | Gly | Cys | | |
| | | 130 | | | | 135 | | | | | 140 | | | | | | |
| GTA | TTG | GAA | GAT | CAA | GAG | CAT | CAA | GTC | GCT | AAA | GAA | GAA | AGG | GAA | ACA | 480 | |
| Val | Leu | Glu | Asp | Gln | Glu | His | Gln | Val | Ala | Lys | Glu | Glu | Arg | Glu | Thr | | |
| | | 145 | | | 150 | | | | | 155 | | | | | 160 | | |
| CCT | ACT | ACT | GAT | AGT | ATG | CCG | CCA | TTA | TTA | CGA | CAA | GCT | ATC | GAA | TTA | 528 | |
| Pro | Thr | Thr | Asp | Ser | Met | Pro | Pro | Leu | Leu | Arg | Gln | Ala | Ile | Glu | Leu | | |
| | | | | 165 | | | | 170 | | | | | | 175 | | | |
| TTT | GAT | CAC | CAA | GGT | GCA | GAG | CCA | GCC | TTC | TTA | TTC | GGC | CTT | GAA | TTG | 576 | |
| Phe | Asp | His | Gln | Gly | Ala | Glu | Pro | Ala | Phe | Leu | Phe | Gly | Leu | Glu | Leu | | |
| | | | 180 | | | | 185 | | | | | | 190 | | | | |
| ATC | ATA | TGC | GGA | TTA | GAA | AAA | CAA | CTT | AAA | TGT | GAA | AGT | GGG | TCT | TAA | 624 | |
| Ile | Ile | Cys | Gly | Leu | Glu | Lys | Gln | Leu | Lys | Cys | Glu | Ser | Gly | Ser | | | |
| | | 195 | | | | 200 | | | | | | 205 | | | | | |

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|
| Met | Ser | Arg | Leu | Asp | Lys | Ser | Lys | Val | Ile | Asn | Ser | Ala | Leu | Glu | Leu | | |
| 1 | | | | 5 | | | | | 10 | | | | | 15 | | | |
| Leu | Asn | Glu | Val | Gly | Ile | Glu | Gly | Leu | Thr | Thr | Arg | Lys | Leu | Ala | Gln | | |
| | | | 20 | | | | | 25 | | | | | 30 | | | | |
| Lys | Leu | Gly | Val | Glu | Gln | Pro | Thr | Leu | Tyr | Trp | His | Val | Lys | Asn | Lys | | |
| | | 35 | | | | | 40 | | | | | 45 | | | | | |

- 41 -

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATTCGATA CTCTATCATT GATAGAGTAT CAAGCTTATC CCTATCAGTG ATAGAGATAC 60
CGTCGACCTC 70

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 49 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTCTATCAT TGATAGAGTT ACTATTAA TCCCTATCAG TGATAGAGA 49

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 71 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGAATTCGAT ACTCTATCAT TGATAGAGTA TCAAGCTTAT CCCTATCAGT GATAGAGATA 60
CCGTCGACCT C 71

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 624 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..624

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG 48
 Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu
 1 5 10 15
 CTT AAT GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG 56

- 44 -

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 74 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTATTATGTT TTATGTTACT GTAAAAGATG TAAAGAGAGG CACGTGGTTA AGCTCTCGGG 60
GTGTGGACTC CACC 74

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 73 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCCCCAAGC ATAAACCCTG GCGCGCTCGC GGCCCCGGCAC TCTTCTGGTC CCCACAGACT 60
CAGAGAGAAC CCA 73

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 74 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAGGCAGCAG GCATATGGGA TGGGATATAA AGGGGCTGGA GCACTGAGAG CTGTCAGAGA 60
TTTCTCCAAC CCAG 74

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

- 43 -

Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His
 50 55 60
 Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg
 65 70 75 80
 Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly
 85 90 95
 Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr
 100 105 110
 Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu
 115 120 125
 Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys
 130 135 140
 Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr
 145 150 155 160
 Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu
 165 170 175
 Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu
 180 185 190
 Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser
 195 200 205

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 92 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGGCCCTATA AAAAGCGAAG CGCGCGGCGG GCGGGAGTCG CTGCGTTGCC TCGCCCCGT 60
 GCCCCGCTCC GCGCCGCCTC GCGCCGCCCCG CC 92

(2) INFORMATION FOR SEQ ID NO:8:

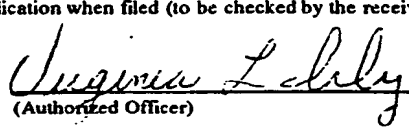
(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGAAGTATA TTAGAGCGAG TCTTTCTGCA CACACGATCA CTTTCCTAT CAACCCCACT 60
 A 61

| MICROORGANISMS | |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
| Optional Sheet in connection with the microorganism referred to on page 38, lines 7-23 of the description * | |
| A. IDENTIFICATION OF DEPOSIT * Further deposits are identified on an additional sheet * | |
| Name of depositary institution * American Type Culture Collection | |
| Address of depositary institution (including postal code and country) * 12301 Parklawn Drive Rockville, MD 10582 US | |
| Date of deposit * August 25, 1993 Accession Number * N/A | |
| B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet <input checked="" type="checkbox"/> | |
| | |
| C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States) | |
| | |
| D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit") | |
| | |
| E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office) <div style="text-align: right;"> (Authorized Officer)</div> <input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau * was _____ (Authorized Officer) | |

Form PCT/RO/134 (January 1981)

ACTCTATCAT TGATAGAGT

19

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTCTATCAA TGATAGAGT

19

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCCCTATCAG TGATAGAGA

19

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTCTATCAC TGATAGGGA

19

WHAT IS CLAIMED IS:

1. A substantially purified and isolated nucleic acid molecule comprising an animal promoter element that comprises a tetR operator sequence.
5
2. The nucleic acid molecule of claim 1 in which the tetR operator sequence is positioned 3' to a TATA-box sequence.
10
3. The nucleic acid molecule of claim 1 in which the promoter element is the PEPCK promoter.
15
4. The nucleic acid molecule of claim 3 in which the tetR operator sequence has been inserted into the NheI site of the PEPCK promoter element.
20
5. The nucleic acid molecule of claim 1, 2, 3 or 4 in which the promoter element controls the expression of a gene of interest.
25
6. The nucleic acid molecule of claim 5 in which the gene of interest is bovine growth hormone.
30
7. A non-human transgenic animal that carries, as a transgene, the nucleic acid molecule of claim 1, 2, 3 or 4.
35
8. A non-human transgenic animal that carries, as a transgene, the nucleic acid molecule of claim 5.
9. A non-human transgenic animal that carries, as a transgene, the nucleic acid molecule of claim 6.

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International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 10582
USAccession No.

N/A

N/A

N/A

N/A

Date of Deposit

August 25, 1993

August 25, 1993

August 25, 1993

August 25, 1993

17. The transgenic animal of claim 15 that is a pig.

5 18. A substantially purified and isolated nucleic acid molecule comprising an optimized tetR gene as depicted in Figure 16.

10 19. The non-human transgenic animal of claim 7 that further carries an optimized transgene encoding the tetR repressor protein and having a sequence as depicted in Figure 16.

15 20. The non-human transgenic animal of claim 8 that further carries an optimized transgene encoding the tetR repressor protein and having a sequence as depicted in Figure 16.

20 21. The non-human transgenic animal of claim 9 that further carries an optimized transgene encoding the tetR repressor protein and having a sequence as depicted in Figure 16.

25 22. A non-human transgenic animal that carries an optimized transgene encoding the tetR repressor protein and having a sequence as depicted in Figure 16.

30 23. A method of selectively inducing the expression of a gene of interest in a non-human transgenic animal comprising administering a tetracycline compound to a non-human transgenic animal that carries a first transgene which is a gene of interest under the control of a promoter element
35 modified to comprise a tetR operator sequence and a second optimized transgene encoding the tetR repressor

10. The non-human transgenic animal of claim 7 that further carries a transgene encoding the tetR repressor protein.

5

11. The non-human transgenic animal of claim 8 that further carries a transgene encoding the tetR repressor protein.

10 12. The non-human transgenic animal of claim 9 that further carries a transgene encoding the tetR repressor protein.

13. A non-human transgenic animal that carries a
15 transgene encoding the tetR repressor protein.

14. A method of selectively inducing the expression of a gene of interest in a non-human transgenic animal comprising administering a
20 tetracycline compound to a non-human transgenic animal that carries a first transgene which is a gene of interest under the control of a promoter element modified to comprise a tetR operator sequence and a second transgene encoding the tetR repressor protein.

25

15. A non-human transgenic animal that carries (i) a first transgene that encodes bovine growth hormone and is under the control of PEPCK promoter element modified to contain a tetR operator at the
30 NheI site; and (ii) a second transgene that encodes tetR repressor protein.

16. The transgenic animal of claim 15 that is a mouse.

35

1/ 19

Figure 1A.

Tn10 Operator sites in Tn10.

Operator 1
 TTGACACTCTATCATTTGATAGAGTTATTTTACCACCTCCCTATCAGTGATAGAGAAAAGT
 Operator 2

Oligonucleotides

General purpose operator.

EcoR1 Operator 1 Hind3 Operator 2 AccI
 GAATTCGAT ACTCTATCATTTGATAGAGT ATCAAGCTTA TCCCTATCAGTGATAGAGA TACCGTGGACCTC

PEPCK-TATA box operator.

Operator 1 Operator 2
 ACTCTATCATTTGATAGAGT TACTATTTTAA TCCCTATCAGTGATAGAGA

protein and having a sequence as depicted in Figure 16.

5 24. A non-human transgenic animal that carries
(i) a first transgene that encodes bovine growth
hormone and is under the control of PEPCK promoter
element modified to contain a tetR operator at the
NheI site; and (ii) a second optimized transgene that
10 encodes tetR repressor protein that has a sequence as
depicted in Figure 16.

25. The transgenic animal of claim 24 that is a
mouse.

15 26. The transgenic animal of claim 24 that is a
pig.

20

25

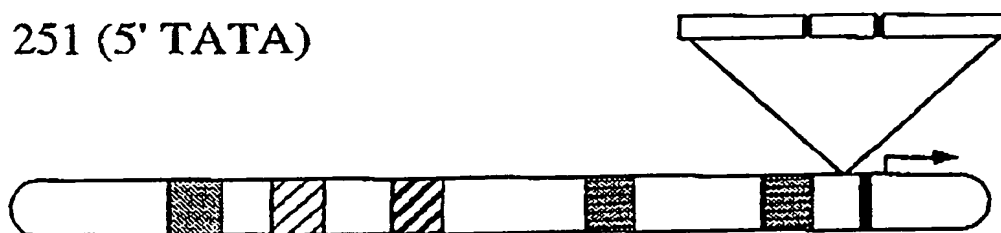
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35

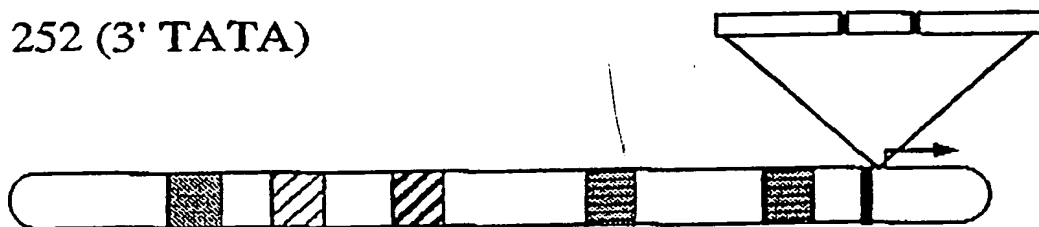
FIGURE 2.

MODIFIED PEPCK PROMOTERS

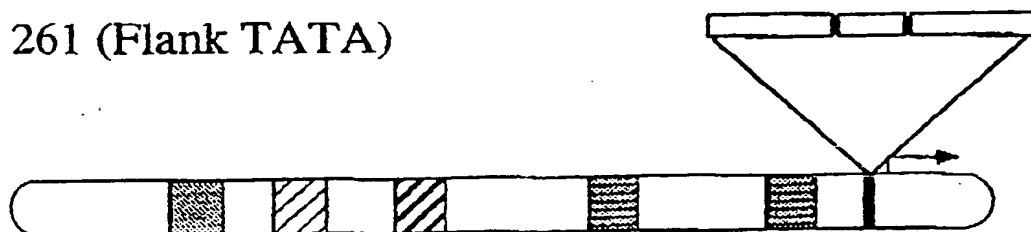
251 (5' TATA)



252 (3' TATA)



261 (Flank TATA)

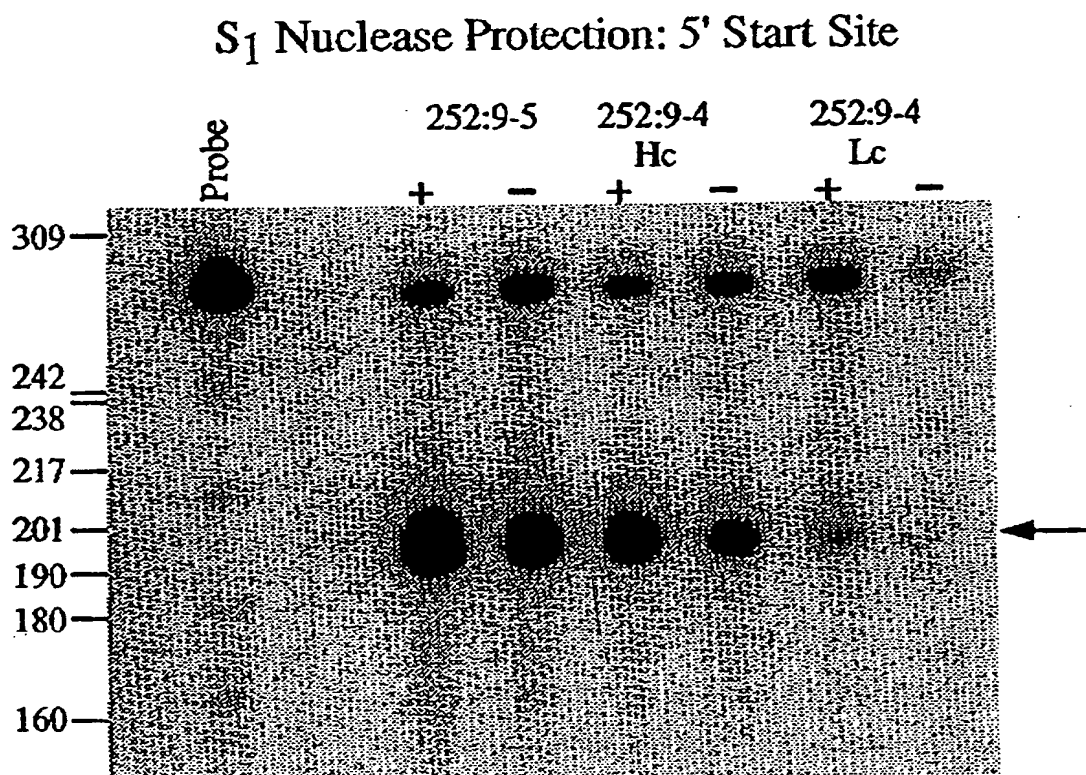


EcoR1 OP1 linker
ggaattcgat-ACT CTA TCA TTG ATA GAG TAT CAA GCT TAT CCC

 OP2 AccI
TAT CAG TGA TAG AGA-taccgtcgacctc

Figure 1B.

FIGURE 4.



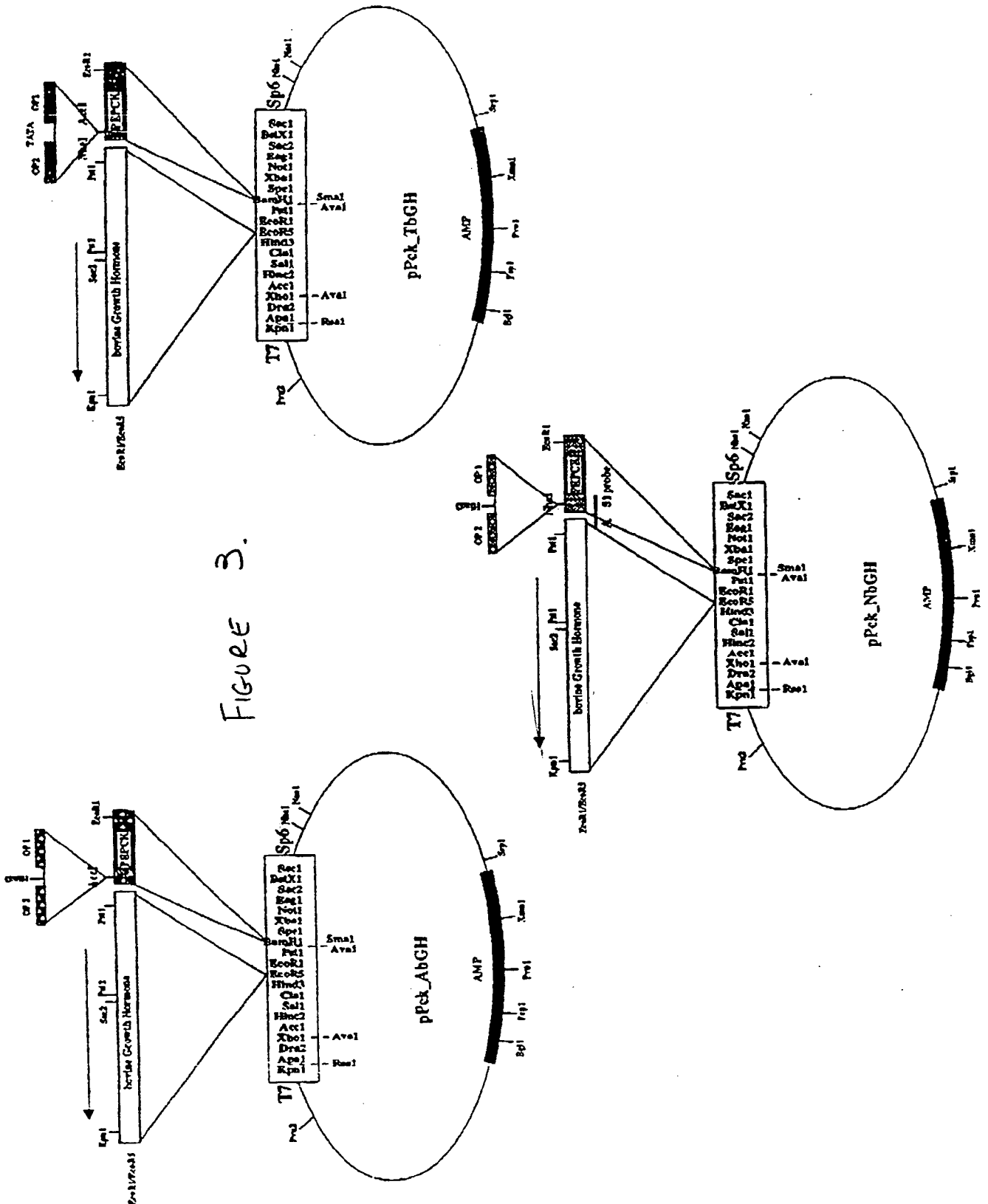


FIGURE 5 (continued)

320 330 340 350 360
 * * * * *
 ACA GAA AAA CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA
 T E K Q Y E T L E N Q L A F L>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1____>
 b b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <1210_a_a_1200_903 TO 1526 OF TRN10TETR_0_a_a_1170a_

370 380 390 400
 * * * *
 TGC CAA CAA GGT TTT TCA CTA GAG AAT GCA TTA TAT GCA CTC AGC
 C Q Q G F S L E N A L Y A L S>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1____>
 b b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <a_1160_a_a_903 TO 1526 OF TRN10TETR_a_1130_a_a_

410 420 430 440 450
 * * * * *
 GCT GTG GGG CAT TTT ACT TTA GGT TGC GTA TTG GAA GAT CAA GAG
 A V G H F T L G C V L E D Q E>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1____>
 b b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <1120_a_a_1110_903 TO 1526 OF TRN10TETR_0_a_a_1080a_

460 470 480 490
 * * * *
 CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA CCT ACT ACT GAT AGT
 H Q V A K E E R E T P T T D S>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1____>
 b b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <a_1070_a_a_903 TO 1526 OF TRN10TETR_a_1040_a_a_

500 510 520 530 540
 * * * * *
 ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA
 M P P L L R Q A I E L F D H Q>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1____>
 b b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <1030_a_a_1020_903 TO 1526 OF TRN10TETR_0_a_a_990a_

550 560 570 580
 * * * *
 GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC
 G A E P A F L F G L E L I I C>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1____>
 b b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <a_980_a_a_903 TO 1526 OF TRN10TETR_a_950_a_a_

590 600 610 620
 * * * *
 GGA TTA GAA AAA CAA CTT AAA TGT GAA AGT GGG TCT TAA
 G L E K Q L K C E S G S *>
 TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON____>
 b b TETR REPRESSOR MRNA [SPLIT] b b b b b>
 <940_a_a_903 TO 1526 OF TRN10TETR_910_a_a_

FIGURE 5.

```

      10      20      30      40
      *      *      *      *
ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG
M  S  R  L  D  K  S  K  V  I  N  S  A  L  E>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<__a__1520__a__a__903 TO 1526 OF TRN10TETR__a__1490__a__a__

      50      60      70      80      90
      *      *      *      *      *
CTG CTT AAT GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC
L  L  N  E  V  G  I  E  G  L  T  T  R  K  L>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<1480__a__a__1470__903 TO 1526 OF TRN10TETR__0__a__a__1440a__

      100     110     120     130
      *      *      *      *
GCC CAG AAG CTA GGT GTA GAG CAG CCT ACA TTG TAT TGG CAT GTA
A  Q  K  L  G  V  E  Q  P  T  L  Y  W  H  V>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<__a__1430__a__a__903 TO 1526 OF TRN10TETR__a__1400__a__a__

      140     150     160     170     180
      *      *      *      *      *
AAA AAT AAG CGG GCT TTG CTC GAC GCC TTA GCC ATT GAG ATG TTA
K  N  K  R  A  L  L  D  A  L  A  I  E  M  L>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<1390__a__a__1380__903 TO 1526 OF TRN10TETR__0__a__a__1350a__

      190     200     210     220
      *      *      *      *
GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG GAA AGC TGG
D  R  H  H  T  H  F  C  P  L  E  G  E  S  W>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<__a__1340__a__a__903 TO 1526 OF TRN10TETR__a__1310__a__a__

      230     240     250     260     270
      *      *      *      *      *
CAA GAT TTT TTA CGT AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA
Q  D  F  L  R  N  N  A  K  S  F  R  C  A  L>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<1300__a__a__1290__903 TO 1526 OF TRN10TETR__0__a__a__1260a__

      280     290     300     310
      *      *      *      *
CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT TTA GGT ACA CGG CCT
L  S  H  R  D  G  A  K  V  H  L  G  T  R  P>
__TETRACYCLINE REPRESSOR PROTEIN (TETR); CODON_START=1__>
__b__b__b__TETR REPRESSOR MRNA [SPLIT]__b__b__b__b__>
<__a__1250__a__a__903 TO 1526 OF TRN10TETR__a__1220__a__a__
```

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FIGURE 7.

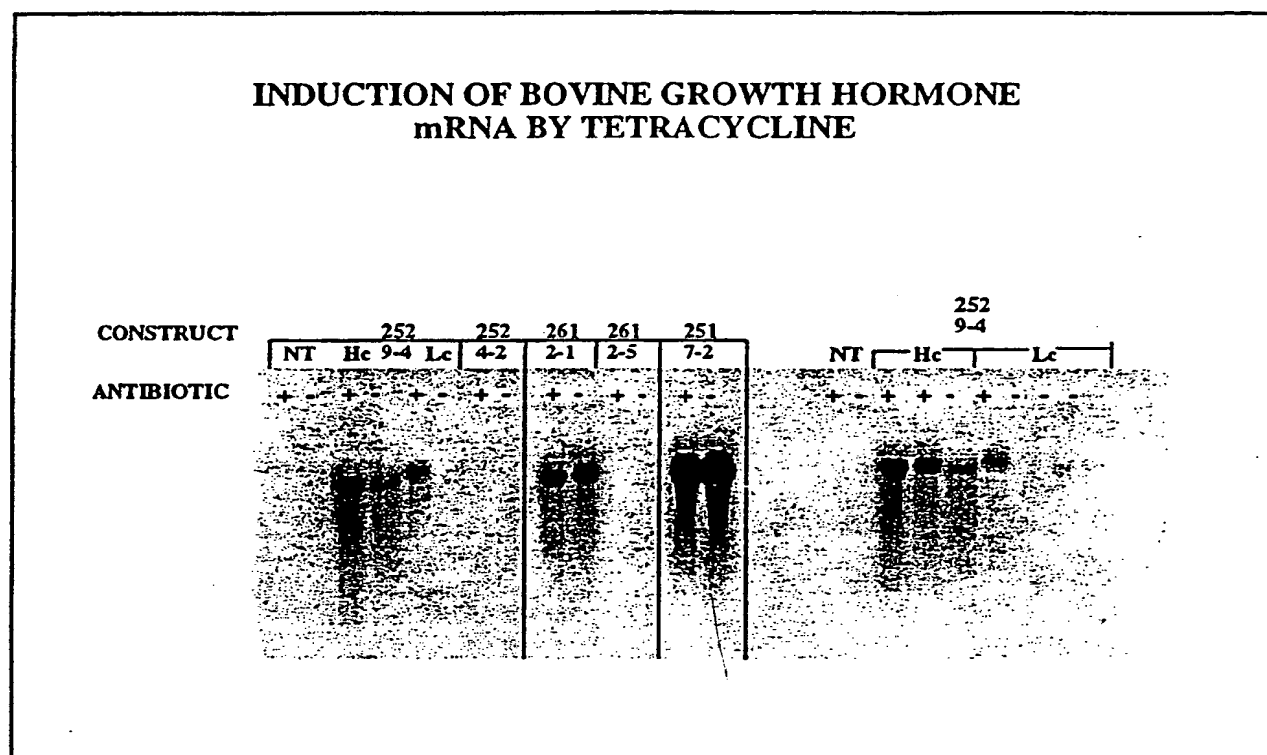


Figure 6.

Mouse Cardiac Myosin Heavy Chain *
TAGGCAGCAGGCATATGGATGCGATATAAGGGCTGGAGCACTGAGAGCTGTCAGAGATTTCTCCAAACCCAG
(Heart)
Tanigawa G, Jarcho JA, Kass S, Solomon SD, Vosberg H.-P, Seidman JG, and Seidman CE. (1990) Cell 62; 991 - 998.

FIGURE 9

*Tissue Specificity. and Tetracycline Induction
of bGH in Line 10-2*

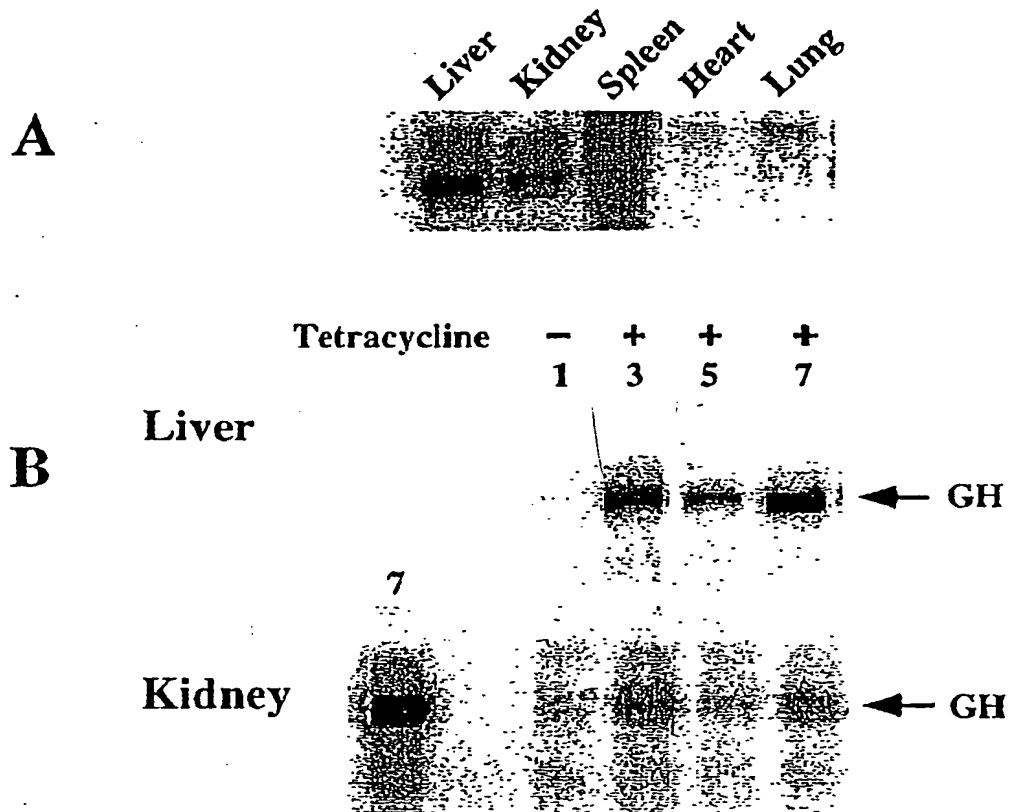
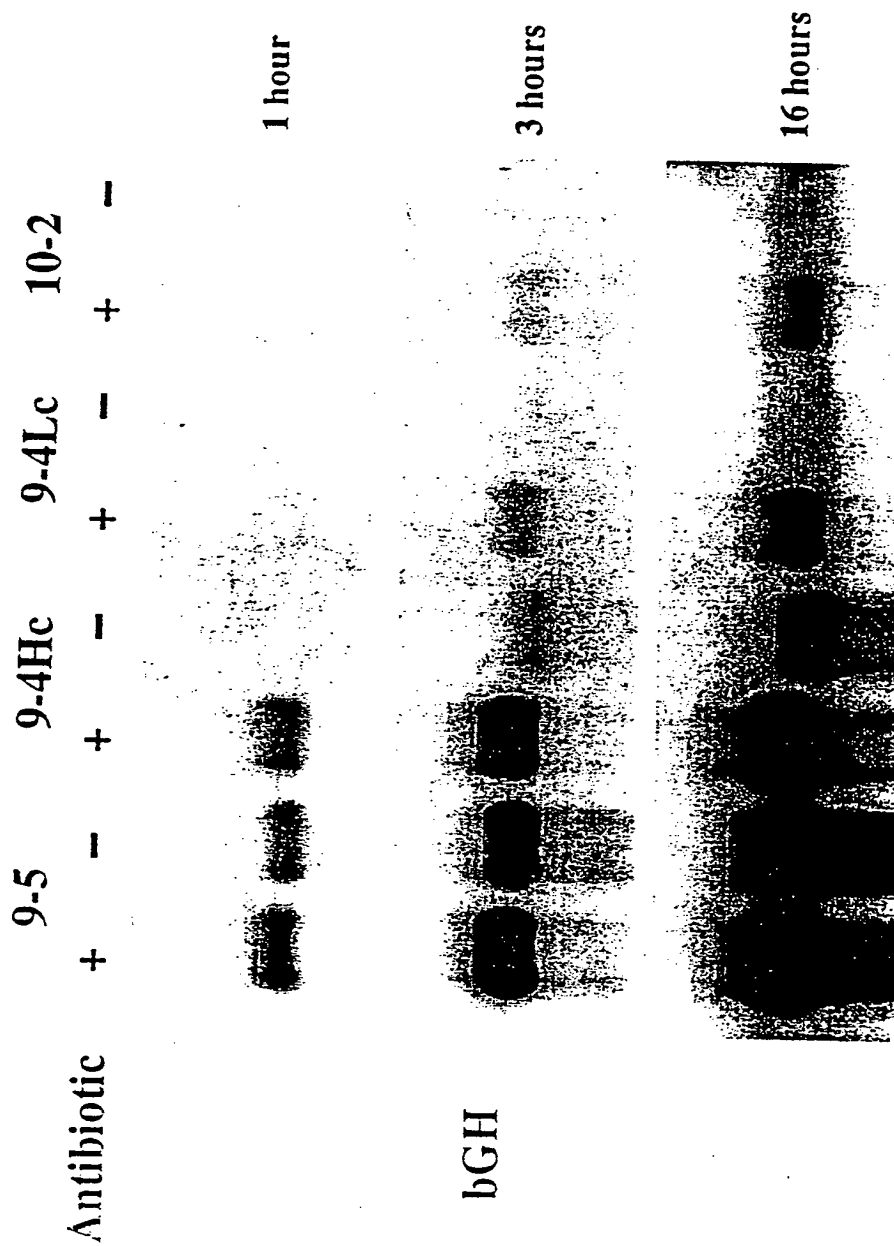



Figure 8.



*Figure 11**Induction of bGH in Construct 345 Offspring*

| FOUNDER | 32 | 33 | 31 | | | | 14 | 19 | 63 | 63 | 44 | 54 | | |
|------------------------------------------------------------------------------------|----|----|----|---|---|---|----|----|----|----|----|----|---|---|
| TETRACYCLINE | - | + | - | + | - | - | + | + | - | + | - | + | - | + |
|  | | | | | | | | | | | | | | |

*Figure 10**345 Repressor Construct*

*FIGURE 13***5' Structure of tetR mRNA**

202 203 210 301 304

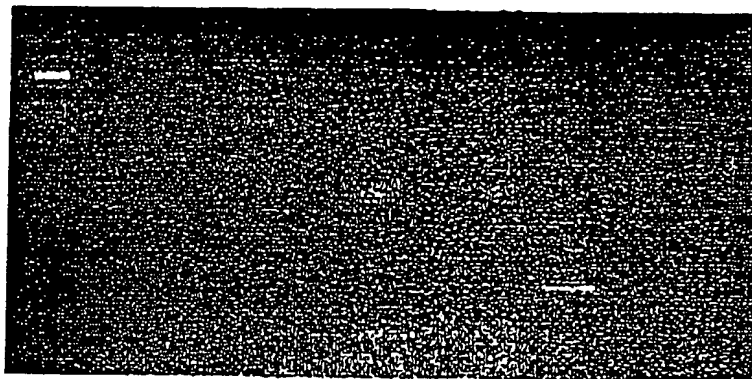
**5' primer**TZ-4
TZ-1TZ-4
TZ-3TZ-4
TZ-3TZ-4
TZ-3TZ-4
TZ-3**3' primer**TZ-3
TZ-4TZ-3
TZ-4TZ-3
TZ-4TZ-3
TZ-4TZ-3
TZ-4

FIGURE 12

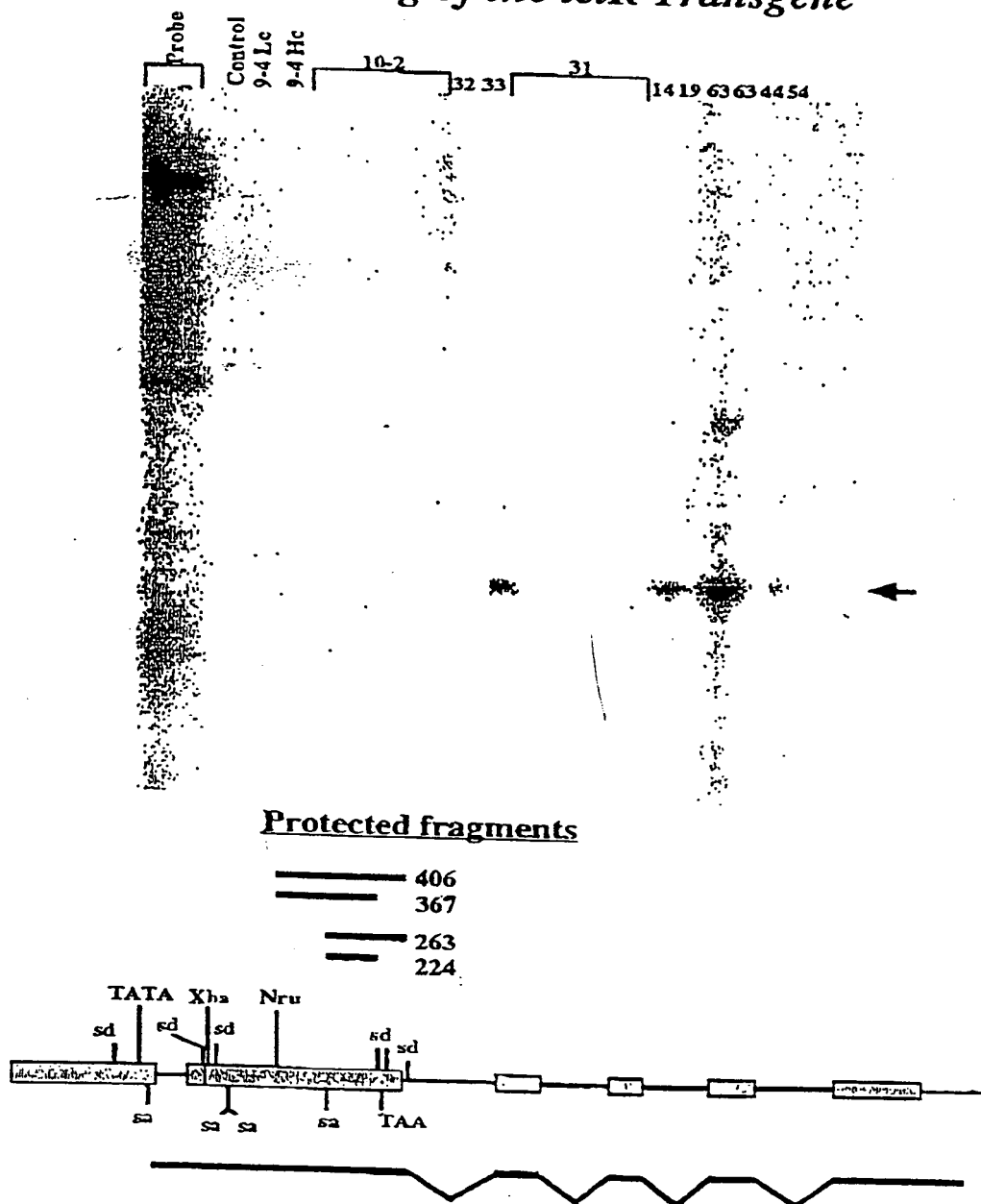
Expression and Alternative Processing of the tetR Transgene

FIGURE 15

Synthetic tetR Component Sequences

LT-1

EcoR5 and EcoR1

GATATCGAATTCATGAGTAGATTGGACAAGAGCAAAGTGATCAATAGTGC
TCTGGAGCTGTTGAATGAAGTGGGCATAGAAGGTCTGACTACCAGAAAGC
TGGCCAGAAAGCTGGGAGTGGAGCAGCCAACATTGTACTGGCATGTGAAG
AATAAGAGGGCTCTGCTGGATGCATTGGCGGTACCAGGC
NsiI KpnI

LT-2

KpnINsiI

GCTCGGTACCTGGATGCATTGGCCATTGAGATGCTGGACAGACACCATAC
ACACTTCTGCCCACTGGAAGGCGAGAGTTGGCAGGACTTCCTGAGGAACA
ATGCTAAGAGTTTCAGATGTGCTCTGTTGAGCCACAGAGACGGTGCTAAA
GTGCACCTGGAATTCGAGC
ApaI EcoRI

LT-3

EcoRIApaI

GCTCGAATTCAAAGTGCACCTGGGTACAAGGCCAACAGAGAAACAGTACG
AGACCCTGGAGAACCAGCTGGCATTCTGTGCGCAACAAGGCTTCAGCCTG
GAGAATGCATTGTATGCTCTGAGTGCTGTGGGTCACTTCACACTGGGTTG
TCTCCTGGAGGACCAGGAGCACCAGGTGGCCAAGGAGGAGGGGAGACCC
CAACCACTGACAGCATGCCCCGGATCCGAGC
SphI BamHI

LT-5

BamHISphI

GCTCGGATCCACAGCATGCCCCATTGCTGAGACAGGCCTATGAGCTGTT
TGACCACCAAGGGGCAGAGCCTGCTTTTCTGTTTGGCCTGGAGCTCATCA
TCTGTGGTCTGGAGAAGCAGCTGAAGTGTGAGAGTGGCTCCTGAAGCTTG
ATATC Hind3/EcoR5

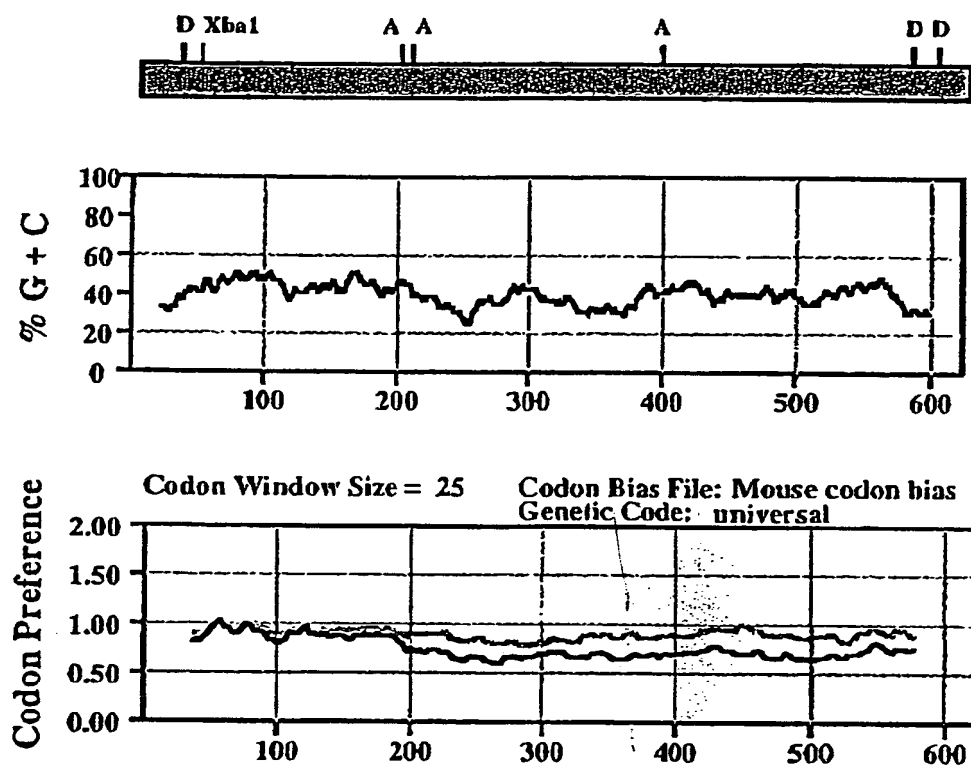
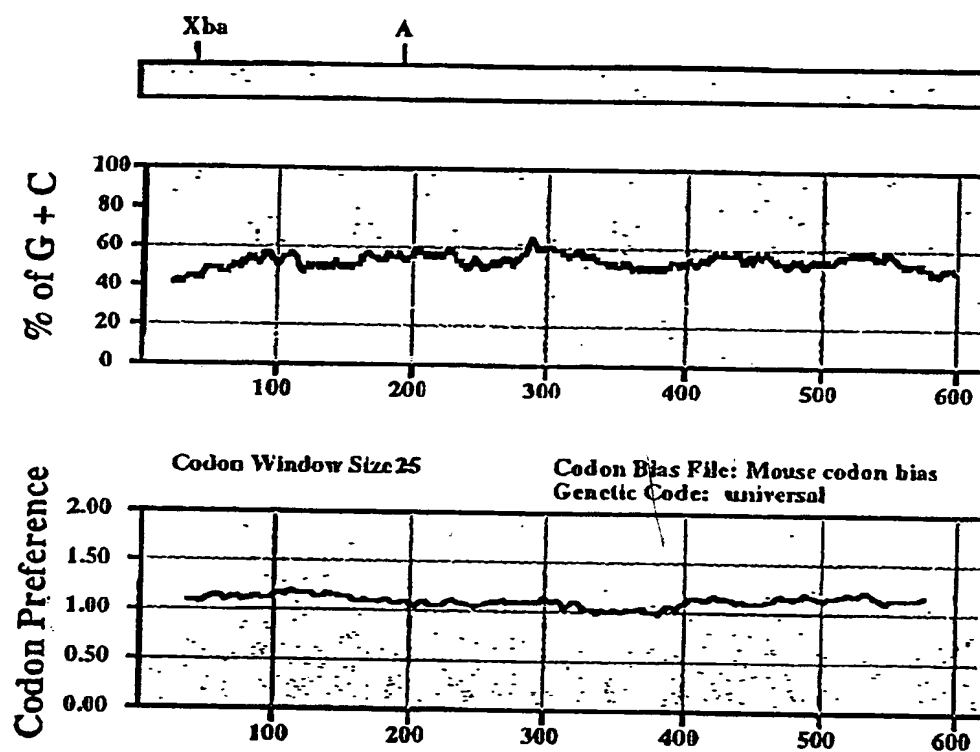
*FIGURE 14**Composition Analysis of Wild Type Tn10 tetR Gene.*

FIGURE 17

Compositional analysis of Synthetic *tetR*



*FIGURE 16***Sequence of Synthetic tetR Gene.**

| | | | |
|------------|------------|------------|------------|
| GATATCGAAT | TCATGAGTAG | ATTGGACAAG | AGCAAAGTGA |
| TCAATAGTGC | TCTGGAGCTG | TTGAATGAAG | TGGGCATAGA |
| AGGTCTGACT | ACCAGAAAGC | TGGCCCAGAA | GCTGGGAGTG |
| GAGCAGCCAA | CATTGTACTG | GCATGTGAAG | AATAAGAGGG |
| CTCTGCTGGA | TGCATTGGCC | ATTGAGATGC | TGGACAGACA |
| CCATACACAC | TTCTGCCCAC | TGGAAGGCGA | GAGTTGGCAG |
| GACTTCCTGA | GGAACAATGC | TAAGAGTTTC | AGATGTGCTC |
| TGTTGAGCCA | CAGAGACGGT | GCTAAAGTGC | ACCTGGGTAC |
| AAGGCCAACA | GAGAAACAGT | ACGAGACCCT | GGAGAACCAG |
| CTGGCATTTC | TGTGCCAACA | AGGCTTCAGC | CTGGAGAATG |
| CATTGTATGC | TCTGAGTGCT | GTGGGTCACT | TCACACTGGG |
| TTGTGTCCTG | GAGGACCAGG | AGCACCAGGT | GGCCAAGGAG |
| GAGAGGGAGA | CCCCAACCAC | TGACAGCATG | CCCCCATTGC |
| TGAGACAGGC | CATAGAGCTG | TTTGACCACC | AAGGGGCAGA |
| GCCTGCTTTT | CTGTTTGGCC | TGGAGCTCAT | CATCTGTGGT |
| CTGGAGAAGC | AGCTGAAGTG | TGAGAGTGGC | TCCTGAAGCT |
| TGATATC | | | |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08230

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y | The Plant Journal, Volume 2, Number 3, issued 1992, Gatz et al., "Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants", pages 397-404, see the entire document. | 1-26 |
| Y | Proc. Natl. Acad. Sci. USA, Volume 89, issued June 1992, Gossen et al., "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters", pages 5547-5551, see the entire document. | 1-26 |
| Y | Proc. Natl. Acad. Sci. USA, Volume 86, issued July 1989, Byrne et al., "Multiplex gene regulation: a two-tiered approach to transgene regulation in transgenic mice", pages 5473-5477, see the entire document. | 1-26 |
| Y | Proc. Natl. Acad. Sci. USA, Volume 88, issued February 1991, Ornitz et al., "Binary system for regulating transgene expression in mice: targeting <u>int-2</u> gene expression with yeast <u>GAL4/UAS</u> control elements", pages 698-702, see the entire document. | 1-26 |
| Y | Proc. Natl. Acad. Sci. USA, Volume 85, issued March 1988, Gatz et al., "Tn10-encoded <u>tet</u> repressor can regulate an operator-containing plant promoter", pages 1394-1397, see the entire document. | 1-26 |
| Y | Nucleic Acids Research, Volume 16, Number 4, issued 1988, Khillan et al., "Gene transactivation mediated by the TAT gene of human immunodeficiency virus in transgenic mice", pages 1423-1430, see the entire document. | 1-26 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08230**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C12N 15/00; C07H 21/00

US CL : 800/2; 435/172.3, 320.1; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/172.3, 320.1; 536/27; 514/152; 935/40, 43, 111

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| <u>X</u> Y | US,A, 5,075,229 (Hanson et al.) issued 24 December 1991, see entire document. | <u>7-9</u> 1-6, 10-26 |
| Y,P | US,A, 5,221,778 (Byrne et al.) issued 22 June 1993, see entire document. | 1-26 |
| Y | Mol. Gen. Genet., Volume 227, Number 2, issued June 1991, Gatz et al., Regulating a modified CaMV 35S promoter by the Tn10-encoded Tet repressor in transgenic tobacco", pages 229-237, see the entire document. | 1-26 |



Further documents are listed in the continuation of Box C.



See patent family annex.

| | | |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
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Date of the actual completion of the international search

15 OCTOBER 1993

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08230

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG (files 154, 55, 312)

U.S. Automated Patent System (file USPAT, 1975-1993)

Search terms: tetracycline, tetR, operator, repressor, binary, transactivate, PEPCK, 6GH, transgenic, mice, transresponder, Tn10, tet, inventor's name.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08230

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)

Group I, claims 1-6 and 18, drawn to a nucleic acid molecule, classified in Class 536, subclass 27, for example.

Group II, claims 7-17 and 19-26, drawn to transgenic animals and a method of using the same, classified in Class 800 subclass 2, for example.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
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